Role of Mu-Opioid Receptors in the Behavioral Effects of the Antidepressant Tianeptine

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Abstract

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For over half a century, the monoamine hypothesis has been the dominant theoretical framework guiding depression research and drug development. This hypothesis posits that depression arises from a deficiency in the monoaminergic neurotransmitters serotonin, norepinephrine, and possibly dopamine, and that antidepressants function by increasing extracellular availability of these monoamines in the brain, especially at the synaptic level. It is clear however, that the monoamine hypothesis cannot fully explain either the pathophysiology of depression nor the mechanisms of antidepressant action.

Tianeptine is an atypical antidepressant used in Europe to treat patients who respond poorly to selective serotonin reuptake inhibitors (SSRIs). The recent discovery that tianeptine is a mu opioid receptor (MOR) and delta opioid receptor (DOR) agonist has provided a potential avenue for expanding our understanding of antidepressant treatment beyond the monoamine hypothesis. This dissertation aims to understand the neural circuits underlying tianeptine's antidepressant effects.

We first characterized the acute and chronic effects of tianeptine on depressive-like and other opioid-related behaviors in mice, and used genetic and pharmacological models to test whether these behavioral effects are mediated by MOR and/or DOR. We found that acute tianeptine administration produced an antidepressant-like reduction in immobility time in the forced swim test, as well as classic opioid-like effects including analgesia, hypophagia, hyperactivity, and conditioned place preference. These behavioral responses to tianeptine are

abolished in MOR knockout (KO) mice and in mice that have been pretreated with an MOR antagonist. By contrast, all responses to tianeptine remained intact in DOR KO mice. Remarkably, unlike other classic opiates such as morphine, chronic tianeptine treatment did not produce tolerance to tianeptine's analgesic effect, nor naloxone-precipitated withdrawal.

The acute behavioral effects of tianeptine (excluding analgesic effects, which were present at 15 minutes, but not 1 hour) were established to occur at 1 hour post-injection and to be largely absent by 3 hours post-injection. Chronically, tianeptine produced an antidepressant effect in corticosterone-treated mice, and prevented the development of restraint-stress-induced depression-like behavior, both in an MOR-dependent manner. Interestingly, tianeptine's chronic antidepressant-like effects were evident in mice after as little as one week of treatment, rather than several weeks as might be expected for SSRIs.

Using tissue-specific MOR knockouts, we further showed that MOR expression on GABAergic cells, specifically somatostatin-positive neurons, is necessary for the acute and chronic antidepressant-like responses to tianeptine. By contrast, tianeptine's behavioral effects did not require the expression MORs on D1- and parvalbumin-expressing cells, nor the expression of ß-arrestin 2. These experiments also revealed a dissociation between the antidepressant-like phenotype and other opioid-like phenotypes resulting from acute tianeptine administration such as analgesia, conditioned place preference, and hyperlocomotion.

Critically, we found that tianeptine's mechanism of action is distinct from fluoxetine in three important aspects: 1) tianeptine requires MORs but not DORs for its chronic antidepressant-like effect, while fluoxetine is the opposite, 2) unlike fluoxetine, tianeptine does not promote hippocampal neurogenesis, and 3) tianeptine's effects appear to persist even after serotonin depletion.

Taken together, these results suggest a novel entry point for understanding what circuit dysregulations may occur in depression, as well as possible targets for the development of new classes of antidepressant drugs.

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Acknowledgments

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Dedication

To my father, who inspired me to be a scientist.

Chapter 1: Introduction

1.1 The Monoamine Hypothesis

Major depressive disorder (MDD) is one of the most common and devastating psychiatric illnesses in the world today, affecting 350 million people and causing two thirds of all deaths by suicide[1]. This high prevalence and mortality rate, combined with the chronic, recurrent nature of the disease, make depression the ninth leading cause of death and disability worldwide[1]. In the United States alone, the NIMH estimates that 16 million adults (6.7% of the population) had at least one major depressive episode in 2015.

In the 1950s, the serendipitous discovery of the antidepressant effects of tricyclics and monoamine oxidase inhibitors (MAOIs)—both drugs with monoaminergic actions—revolutionized the study and treatment of depression by leading to the development of the monoamine hypothesis[2-4]. This hypothesis posits that depression arises from a deficiency in the monoaminergic neurotransmitters serotonin, norepinephrine, and possibly dopamine, and that antidepressants function by increasing extracellular availability of these monoamines in the brain, especially at the synaptic level[5]. What followed was an era of rational drug design for depression that culminated in the advent of selective serotonin reuptake inhibitors (SSRIs), which have since replaced both tricyclic and MAOI antidepressants as the first-line treatment for depression[6].

It is clear however, that the monoamine hypothesis cannot fully explain either the pathophysiology of depression nor the mechanisms of antidepressant action, given the absence of robust mood changes following serotonin depletion, especially in patients with untreated depression[7], and the mismatch in time course between the chemical and therapeutic effects of most antidepressants[8]. Moreover, monoaminergic antidepressants have a limited efficacy:

about a third of depressed patients do not remit to treatment with monoaminergic drugs[9], and even those who do often experience cumbersome side effects such as sedation, cardiovascular issues, and cognitive impairment for tricyclics[10], and sexual dysfunction, weight gain, and sleep disturbance for selective serotonin reuptake inhibitors (SSRIs)[11-13]. Consequently, there has been a pronounced shift in research focus in the last few decades toward other neurochemical systems that could also be dysregulated in depression. In particular, the glutamatergic and opioid systems are emerging as key players in mood and anxiety disorders[14,15].

1.2 The Opioid System and Depression

1.2.1 History

Although opioids are most commonly known for their analgesic effects, the use of opioid drugs to treat psychiatric illnesses such as depression and anxiety dates back thousands of years. The Sumerians were known to have cultivated poppies (*Papaver somniferum*) as early as 3400 BC, and produced raw opium by lancing the poppy seed pods and drying the exuded paste[16,17]. The antidepressant and anxiolytic effects of opium were so effective that the Sumerians referred to the poppy as “Hul Gil” or “the joy plant”[16-19]. Egyptian and Assyrian sources such as the Ebers papyrus (circa 1500 BC) and the Assyrian Medical Tablets (seventh century BC) repeatedly mention medical preparations of the opium poppy, and in the *Odyssey*, Homer refers to nepenthes, a substance of Egyptian origin given as a remedy against grief, which scholars generally believe contained opium[18,19].

In the early 16th century, the Swiss physician Theophrastus Parcelus (1493–1541) reintroduced opium for medical use in Western Europe. He discovered that opium alkaloids are more soluble in alcohol then in water, allowing him to formulate his namesake laudanum, a

tincture of opium that could be taken orally, paving the way for regimented use of opiate drugs in medicine[19,20]. In Germany, between 1750 and 1910, the Engelken family developed and published a protocol for treating of melancholic states marked by simultaneous depression and agitation, using opium for its unique combination of sedative and activating effects—an approach that established opium preparations as the most important method of psychiatric treatment for over a century[20]. By the late 19th to early 20th century, Emil Kraepelin, a seminal figure in modern psychiatry, introduced a regimen for treating of "states of dysphoria" and "agitated depression" in which a tincture of opium was given daily in escalating doses, gradually weaned, and then finally discontinued, over the course of about two months[21]. This "opium cure" was reported to be effective as a treatment for depression in several anecdotal reports and contemporary psychiatric tests, and was widely used during the late 19th to mid 20th centuries[22].

Although opium and its derivatives effectively treated symptoms of depression and anxiety, their high potential for abuse and dependency meant they were quickly abandoned following the discovery of monoaminergic antidepressants such as MAOIs and TCAs in the 1950s, and SSRIs by the late 1980s[21,22]. However, despite the plethora of monoamine-based pharmacotherapies for depression available today, the incidence of treatment-resistant depression remains high, and there exists a dire need for new clinical approaches in this setting[23]. In recent years, opiate drugs have re-emerged as a viable treatment option for major depression. Not only have a number of pre-clinical studies implicated the opioid system in the etiology of depression and other such mood disorders, in the clinical setting, buprenorphine and other opiates have seen use as a remedy for treatment-resistant depression, and for depression

comorbid with addictive disorders, suggesting there may yet be a therapeutic niche for opioid-based antidepressant drugs[14].

1.2.2 The Endogenous Opioid System

Opiate drugs engage an endogenous neuromodulatory system comprised of three main opioid receptors known as mu (μ), delta (δ), and kappa (κ), as well as the non-opioid receptor, nociceptin/orphanin FQ receptor (NOR)[24]. These opioid receptors belong to the class A gamma subgroup of seven transmembrane G protein–coupled receptors (GPCRs) and show 50–70% homology between their genes, although additional pharmacologic subtypes may result from alternative splicing, post-translational modifications, and/or receptor oligomerization[24-27].

Opioid receptors interact with a family of endogenous opioid peptides known as ß-endorphin, enkephalins, and dynorphins, which are derived from the precursors proopiomelanocortin, proenkephalin, and prodynorphin, respectively[24,27]. ß-endorphin (and morphine-like drugs) primarily acts at μ-opioid receptors (MORs), while Met- and Leu-enkephalins have high affinity for both δ-opioid receptors (DORs) and MORs[24,27]. These ligands act as antinociceptive agents[27]. By contrast, dynorphin and its related peptides can elicit both pro- and antinociceptive effects via N-methyl-d-aspartate (NMDA) receptors and KORs, respectively[27]. Nociceptin/orphanin FQ, the endogenous ligand for NORs, have been associated with pain mechanisms and several behaviors linked to psychological stress in nonclinical studies[28,29]. Both peptides and receptors are expressed throughout the central and peripheral nervous system, in neuroendocrine, ectodermal, and immune cells[27,30].

When activated by an agonist (whether endogenous opioid peptides or exogenous drugs), opioid receptors convey extracellular stimulation to multiple downstream effector proteins, second messengers, and ion channels via G-protein dependent and independent (e.g. ß-arrestin signaling) signaling pathways[24,27]. Dissociation of G-protein subunits inhibits the activity of adenylate cyclases, activates G protein-coupled inwardly-rectifying potassium channels (GIRKs), and inhibits voltage gated calcium channels[24,27]. These actions collectively reduce neuronal excitability in opioid receptor-expressing cells through decreased Ca2+-dependent neurotransmitter release, and pre- and post-synaptic hyperpolarization[31].

Independent of G-protein activation, activated opioid receptors can also become phosphorylated at serine and threonine residues at their c-terminus by G protein-coupled receptor kinases (GRKs), protein kinase A (PKA), or protein kinase C (PKC), thereby increasing receptor affinity for the effector proteins ß-arrestin 1 and ß-arrestin 2[32]. The recruitment of ß-arrestin prevents further G protein signaling and targets the opioid receptor for internalization, after which it undergoes either dephosphorylation and return to the cell surface, or degradation[32]. ß-arrestin recruitment and receptor internalization is believed to be a crucial factor in the development of analgesic tolerance, physical dependence, and other adaptive changes that stem from prolonged receptor activation[31,33]. In addition to their classic roles in desensitization and internalization, ß-arrestins have also recently been found to act as signal transduction scaffolds for many pathways, particularly those of the mitogen activated protein kinases[32].

Notably, different agonists binding to the same GPCR can stabilize the receptor in multiple conformations, resulting in differential activation of cell signaling pathways and, ultimately, divergent physiological outcomes[34]. This phenomenon, known as biased agonism, is particularly exciting in the context of opioid research, as selective control of downstream

signaling may provide an avenue for dissociating the therapeutic benefits of opioids (e.g. analgesia and antidepressant efficacy) from their negative side effects (e.g. opioid dependence, tolerance, and respiratory depression for MOR agonists).

One prominent hypothesis has posited that the side effect profile of MOR-based drugs may be attributed to ß-arrestin 2 (rather than G protein) signaling. This was based on a series of seminal preclinical studies showing that genetic knockout or siRNA knockdown of ß-arrestin 2 enhances acute analgesia, reduces tolerance, attenuates physical dependence, and decreases respiratory depression and constipation in response to morphine, the prototypical small molecule MOR agonist[35-37]. Much research has subsequently been devoted to developing G protein-biased MOR agonists that preferentially signal via the canonical G protein pathway, while further minimizing β-arrestin 2 recruitment and signaling[34]. In fact, one such G protein-biased agonist, oliceridine (TRV130), has proceeded to Phase III clinical trials[38] and was recently approved in the United States to treat acute pain[39].

This presents something of a paradox, however, as morphine is a G protein biased agonist that exhibits high efficacy for G protein activation, but little arrestin recruitment[40]. Indeed, a growing body of evidence seems to contradict the notion that G protein-biased agonism at MOR will provide substantially improved therapeutic profile: later studies[41-43] have failed to replicate the early results in ß-arrestin 2 knockout mice implicating the arrestin pathway in opioid-induced side effects[35-37], and "G protein-biased" mutant MOR mice (which express a phosphorylation-deficient mutant MOR that does not recruit ß-arrestins) have recently been shown to undergo respiratory depression in response to both morphine and fentanyl[44].

As such, it appears that there may not be a straightforward correlation between G protein vs. β-arrestin bias in various opioids and their therapeutic profile. Nevertheless, biased agonism

remains an intriguing lens through which to approach the study and development of opioid signaling pathways, and closer attention to nuances such as receptor splice variants, ß-arrestin isoforms (ß-arrestin 1 vs. ß-arrestin 2), and variability in receptor desensitization and internalization, may yet yield advances in the design of biased opioids with improved pharmacological profiles.

1.2.3 Preclinical Evidence of Opioid Involvement in Depression

In addition to their integral role in pain processing, opioids also regulate many other physiological functions, such as stress responses, respiration, gastrointestinal transit, and endocrine/immune functions[30]. More importantly for the context of the present work, a plethora of human and rodent studies have shown that opioid peptides and receptors are highly expressed in limbic and paralimbic brain areas implicated in mood regulation[14,30]. This, combined with the potent euphoric effects of known opiate drugs, establishes the opioid system as a key player in both reward processing and mood control, and thus a logical target for the development of drugs to treat emotional dysfunction.

Indeed, all three major classes of opioid receptors (mu, kappa, delta) have been implicated to some extent in the pathophysiology and treatment of depression. Data from constitutive knockout (KO) mice for MOR, DOR, and KOR suggest that these receptors differentially regulate reward processes and emotional responses via distinct mechanisms[45-49]. Both pharmacological and rodent studies indicate that MORs are involved in reward processing for both drugs of abuse and natural stimuli, including social reward[50]. Mu opioid stimulation has been widely implicated in the positive properties of social behaviors, such as the perception of mother-related stimuli and attachment behavior in infant birds, rodents, dogs, and primates—

including humans[51-59], the regulation of sexual behavior[60-62], and the positive subjective properties of social play behavior in adolescent rats[63,64].

The strong rewarding effects of MOR agonists such as morphine likely contributes to both the success of the "opioid cure" for depression and onset of addictive behaviors[22]. Accordingly, acute pharmacological activation of MOR has been reported to reduce depressive-like behaviors in many behavioral paradigms, such as learned helplessness in rats, and forced swim and tail suspension in mice[14] . Central administration of the peptides endomorphin-1 and endomorphin-2, which selectively bind to MORs[65], decreased the immobility time in the tail suspension and forced swim tests in mice without affecting motor activity[66]. These effects were blocked by the non-selective opioid antagonist naloxone and the MOR selective antagonist funaltrexamine, but not by the DOR and KOR selective antagonists, naltrindole and nor-binaltorphimine (nor-BNI)[66]. Contrary to expectations, however, MOR KO mice actually exhibit decreased anxiety- and depressive-like behaviors[45,46]. There are several explanations that might account for this apparent contradiction. Perhaps constitutive MOR knockout causes mice to compensate by developing an overall higher mood state. Alternatively, acute (i.e., pharmacological treatment) and chronic (i.e., constitutive KO) MOR de-activations could produce opposite antidepressant- and depressant-like effects, respectively.

The manner in which DORs regulate reward processing is less clear. DOR KO mice exhibit a variety of phenotypes, including increased alcohol consumption[67] and decreased nicotine-self administration[68]. Although morphine self-administration is preserved in these mice, conditioned place preference for both morphine and nicotine was decreased[47,68], suggesting that DORs may be involved in contextual learning more so than opioid reward. Unlike their MOR KO counterparts, DOR KO mice exhibit increased anxiety- and depressive-

like behaviors, and additional preclinical studies showing that DOR activation reduces persistent pain and improves negative emotional states[69] further support the hypothesis that the enkephalin-DOR system enhances mood. Consistent with the increased depressive-like behaviors observed in DOR KO mice, DOR agonists have been shown to produce antidepressant-like effects in the learned helplessness, forced swim, tail suspension, and olfactory bulbectomy paradigms for both rats and mice[14].

KOR activation is thought to contribute to negative emotional states and has been shown to antagonize the reinforcing effects of drugs such as cocaine, morphine, heroin, and ethanol[70]. In rodents, kappa agonists produce conditioned place aversion[71-73] and depression-like behavior, as evidenced by forced swim and reward stimulation tests[74-77], whereas KOR antagonists have antidepressant[75,78,79] and anxiolytic effects[80,81]. Unlike MOR agonists, KOR agonists have also been shown to decrease social play in rats[63]. Moreover, the dysphoric components of stress, which appear to be encoded by the dynorphin-KOR system, are thought to contribute to the development of mood and substance abuse disorders[82]. KOR activity is potentiated by stressors such as restraint, social defeat, and repeated forced swim, and helps to mediate stress-induced psychopathology[83]. This may explain why KOR KO mice do not exhibit markedly different depressive- or anxiety-like phenotypes compared to controls in classical behavioral models involving limited stress [45].

1.2.4 Opioid Regulation of Mood in Humans

The opioid system has been shown to regulate mood in humans as well as in laboratory animals. MORs are highly expressed in several brain regions implicated in the response to stressors and emotionally salient stimuli, including cortical areas such as the rostral anterior

cingulate and prefrontal cortex, and subcortical regions including the nucleus accumbens, ventral pallidum, amygdala, thalamus, and insular cortex[14,84]. Some studies have shown that endogenous serum ß-endorphin levels are decreased in depressed patients compared to healthy controls[85-91] while others have found the opposite[92-97], suggesting that that the direction of the changes in plasma ß-endorphin levels may diverge in different subsets of patients defined by factors such as depression type, gender, or presence of comorbid disorders.

There is also evidence suggesting that the therapeutic effects of many antidepressants may be achieved by via modulation endogenous opioids or through gradual changes in opioid receptor expression. Local application of fluoxetine into the nucleus accumbens or the hypothalamic arcuate nucleus caused local release of ß-endorphin[98], and antidepressant treatment with the SSRI fluvoxamine[87] increased plasma beta endorphin levels overall. Naloxone, a preferential mu-opioid receptor antagonist, has been found to inhibit the antidepressant effects of various tricyclic antidepressants in the forced swim test, as well as the effect of morphine and imipramine in learned helplessness[99-102]. Paroxetine, reboxetine, and moclobemide significantly altered MOR binding site density in various brain regions[103], and the antidepressant actions of electro-convulsive therapy have similarly been associated with changes in opioid signaling[104,105]. Notably, the acute antidepressant and anti-suicidal effects of ketamine, a noncompetitive NMDAR antagonist, were also recently shown to be attenuated by opioid receptor antagonism with naltrexone[106,107]. Taken together, these studies demonstrate the potential involvement of endogenous opioid regulation in the therapeutic effects of a variety of drugs with diverse antidepressant mechanisms.

Additionally, the endogenous opioid system is hypothesized to regulate a network of brain regions that protect emotional well-being within a social environment. The A118G

polymorphism of the MOR gene (OPRM1) has been associated with increased dispositional and neural sensitivity to social rejection[108] and greater social hedonic capacity in both adult healthy volunteers and psychiatric patients[109]. Studies using an MOR radiotracer and positron emission tomography to measure *in vivo* changes in MOR availability in human subjects during social rejection and acceptance have found that social rejection significantly activated the MOR system in the ventral striatum, amygdala, midline thalamus and periaqueductal gray[110]. This pattern of activation is similar to that observed during physical pain and supports the hypothesis that neuronal mechanisms for reducing social and physical pain are regulated by overlapping pathways[110]. Social acceptance, by contrast, elicited weaker overall MOR activation, but activation in the nucleus accumbens was associated with an increased desire for social interaction, suggesting that the MOR system may act to both reduce distress and mediate reward in response to social cues[110].

Altered endogenous opioid activity may also be a mechanism for impaired emotion regulation during social rejection and acceptance in major depressive disorder. During rejection, depressed patients exhibit reduced endogenous mu opioid release in brain regions regulating stress, mood and motivation and are slower to emotionally recover compared to healthy controls[111]. Even during acceptance, these patients showed only short-lived increases in positive affect that did not significantly increase self-esteem or social motivation[111]. Thus, altered endogenous MOR signaling in major depressive disorder may simultaneously impede emotional recovery from negative social interactions and decrease the pleasure derived from positive social interactions, thereby contributing to poor treatment outcomes by reinforcing depressive states and triggering relapse.

1.2.5 Clinical Use of Opioids as Antidepressants

The efficacy of opioid drugs as antidepressants is not merely theoretical, as select opioids have already shown promising therapeutic potential in clinical trials for depression. Exogenously administered ß-endorphin has been reported to have antidepressant properties in depressed patients[112-117], and synthetic opioid ligands such as cyclazocine (a mixed opioid agonist/antagonist), oxycodone, and oxymorphone (both MOR agonists), appear to have antidepressant effects in patients suffering from refractory or treatment-resistant depression[118,119]. In particular, buprenorphine (a partial mu opioid receptor agonist and kappa opioid antagonist) has been shown to effectively reduce depression in patients who are resistant to SSRIs and tricyclic antidepressants[120-123] or patients with comorbid substance use disorder[124]. One study looking at the effects of treating depressed patients with a 1:1 dose of buprenorphine and samidorphan (a potent MOR antagonist,) found that the regimen was well tolerated and produced significant antidepressant effects[125]. Similarly, small clinical studies of individuals with treatment-resistant depression have found that the synthetic opioid tramadol (a weak MOR agonist), appears to have antidepressant/anti-suicidal effects[126-128].

1.3 Tianeptine

A particularly exciting example of a clinically-effective opioid-acting drug is tianeptine (Stablon®, Coaxil®, Tatinol®), an atypical antidepressant with structural similarities to the TCAs but with different pharmacological properties[10]. Tianeptine is approved for the treatment of major depressive disorder in Europe, Asia, and South America, and has been used as an effective antidepressant for several decades[10,129,130]. Numerous double-blind, comparative trials evaluating tianeptine in patients with major depression, bipolar depression,

dysthymia, and adjustment disorder have established that patients treated with tianeptine experience significantly reduced symptoms of depression (as measured by the Montgomery-Asberg Depression Rating Scale, or MADRS) and decreased frequency of relapse and recurrence compared to patients given placebo[10,131]. In double-blind clinical studies ranging from 2-24 weeks in length, tianeptine is observed to have similar efficacy compared to many other antidepressants, including amitriptyline[132-134], imipramine[135], paroxetine[136], and fluoxetine[137-140]. Moreover, a metanalysis of studies comparing tianeptine to the SSRIs fluoxetine, paroxetine, and sertraline confirmed that tianeptine's antidepressant efficacy is statistically indistinguishable from that of SSRIs[141].

Additionally, tianeptine may confer distinct advantages over standard monoamine therapies, as it displays an improved side effect profile compared to SSRIs and tricyclics. Tianeptine is generally well tolerated, with the most common adverse side effects being nausea, constipation, abdominal pain, headache, dizziness, and changes in dreaming[10,129,130,142]. Unlike tricyclic antidepressant agents, tianeptine is not associated with sedation, cognitive impairment, or cardiovascular issues, nor is it associated with sexual dysfunction or weight gain[11,142,143]. A 6-week double blind study comparing tianeptine and paroxetine in depressed patients without co-morbid anxiety found that tianeptine was better tolerated and less likely to cause patients to discontinue treatment due to adverse effects[136]. Tianeptine may actually have slight activating effects in the realm of attention, and rapidly alleviates the cognitive and anxiety symptoms of depression[144]. Analysis of individual items in the Montgomery-Asberg Depression Rating Scale (MADRS) shows that decreased ability of concentration and inner tension are more rapidly improved in tianeptine-treated than in fluoxetine-treated patients, with therapeutic onset within one week of treatment, rather than after

the several weeks required for SSRIs[140]. A growing body of research suggests that tianeptine may also be effective in patients underserved by existing treatments, such as the elderly[145], those refractory to SSRI monotherapy[146], and those experiencing depression comorbid with other conditions such as Parkinson's disease[147], post-traumatic stress disorder[148], or alcohol addiction[149].

The therapeutic benefits of tianeptine observed in humans have been robustly recapitulated using stress-based animal models of depression. These studies demonstrate that tianeptine largely counteracts the effects of chronic stress on neuronal structure and plasticity in brain regions strongly associated with both depressive symptoms and antidepressant actions, including the hippocampus, amygdala, and prefrontal cortex. In psychosocially stressed tree shrews, tianeptine treatment reverses many major hippocampal modifications: it antagonizes stress-induced changes in cerebral metabolites such as N-acetylaspartate, inhibits stress-induced reduction in proliferation of the granule precursor cells in the dentate gyrus, and prevents stress-induced decreases in hippocampal volume[150,151]. Similarly, in rodents, tianeptine—but not the SSRIs fluoxetine and fluvoxamine—can prevent and reverse atrophy of the CA3 pyramidal neurons in the hippocampus following 2–3 weeks of repeated stress or corticoid administration[152,153]. Conversely, in the amygdala, where chronic stress increases dendritic length and arborization, tianeptine has been shown to prevent both dendritic hypertrophy and increased anxiety-like behavior following chronic immobilization stress[154,155]. Chronic tianeptine has also been reported to prevent stress-induced potentiation of aggressive conflicts and to reduce fear conditioning, which further suggest that the amygdala may provide a cellular substrate for some of tianeptine's behavioral effects. Tianeptine has also been shown to block the

effects of stress on memory[156] and synaptic plasticity in the hippocampus and prefrontal cortex[157-159].

Tianeptine differs markedly from other antidepressants in that it shows no affinity for monoaminergic neurotransmitter receptors, does not inhibit the uptake of serotonin or norepinephrine in the central nervous system[160,161], and does not inhibit MAOa and MAOb activities in the cortex, hippocampus, or hypothalamus. In fact, early studies suggested that tianeptine might actually decrease extracellular serotonin (5-HT) levels via 5-HT re-uptake enhancement[162,163]. However, the validity of these older studies has since been called into question and more recent work indicates that tianeptine does not significantly impact extracellular levels of 5-HT one way or another, at least in the corticolimbic structures of awake rats[164,165]. Along this same vein, electrophysiological studies also show that chronic tianeptine administration does not appear to affect serotonergic signaling[165].

Indeed, in the past decade or so, theories about tianeptine's mechanism of action have shifted away from serotonin and towards alternatives such as modulation of glutamatergic pathways[130,160,166]. In animal models, tianeptine has been found to inhibit various stress-induced pathological changes in glutamatergic neurotransmission in the hippocampus [160,167,168]. Nevertheless, tianeptine's direct molecular target remained unknown until 2014, when a screen of GPCR binding using the Psychoactive Drug Screening Program showed that tianeptine binds to human MOR with a Ki of 383±183 nM and—to a lesser extent—to human DOR (Ki>10 μM)[169]. Tianeptine did not exhibit any affinity for KOR, nor for any other GPCR, transporter, or ion channel targets[169]. BRET-based assays further demonstrated that tianeptine showed full agonism at mouse MOR for both G-protein activation (EC_{50} =641±120 nM) and downstream inhibition of cAMP accumulation (EC_{50}=1.03±0.10 μM)[169].

Tianeptine was also found to be a DOR agonist—albeit with a potency an order of magnitude lower than MOR—with regards to both G-protein activation (EC_{50}=14.5±6.6 μM) and inhibition of cAMP accumulation (EC_{50}=9.46±1.34 μM), but showed no activity at KOR[169].

1.4 Cell Type and Brain Region Specificity

1.4.1 GABA and Depression

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian central nervous system. Together with excitatory glutamatergic neurons, GABAergic neurons modulate the inhibitory-excitatory balance necessary for proper brain function by regulating cortical firing rate, timing, bursting, rhythms, and synchrony[170]. Numerous studies suggest GABAergic deficits may causally contribute to depressive disorders, while antidepressant therapies may restore normal GABAergic synaptic transmission. Depression is associated with reduced levels of GABA in plasma[171,172], cerebrospinal fluid[173,174], and resected cortical tissue[175], and depressed patients tend to have low GABA in brain regions responsible for emotional-cognitive processes, including the PFC[176], amygdala[177], and anterior cingulate cortex[178]. These reductions were found to be more pronounced in cases of treatment resistant depression[178,179], but normalized in remitted patients[176]. Brain GABA levels in depressed patients are elevated following transcranial magnetic stimulation[180], SSRI treatment, electroconvulsive therapy, and cognitive-behavioral therapy[181-183]. Studies of depressed patients have also shown that depression is accompanied by changes in the subunit composition of the principal receptors ($GABA_A$ receptors) mediating GABAergic inhibition[184-186]

In mice, GABAergic neurotransmission has been found to mediate the rapid antidepressant-like effects of scopolamine[187], and the depressive-like phenotypes of $GABA_A$ receptor mutants can be reversed by treatment with monoaminergic antidepressants, as well as by subanesthetic doses of ketamine[188]. It has been suggested that ketamine and scopolamine produce their rapid antidepressant actions by blocking NMDA and ACh-M receptors, respectively, on tonic firing GABA interneurons, thereby disinhibiting excitatory neurons and causing a glutamate burst that stimulates synaptic changes in the mPFC[187,189], potentially reversing the synaptic deficits observed following stress and depression. It should be kept in mind, however, that there is also some support for the hypothesis that these drugs act directly on glutamatergic neurons[189-191].

Moreover, mounting evidence shows that GABAergic transmission plays an important role in modulating stress, the most important vulnerability factor for depression and other mood disorders[192]. In rats, for instance, maternal separation stress during early life results in both increased stress reactivity and reduced $GABA_A$ receptor ($GABA_ARs$) expression throughout multiple brain areas in adulthood[193], and the depressive-like phenotype induced by inescapable tailshock stress is associated with reduced abundance and function of $GABA_ARs$ in the cerebral cortex[194]. Increased secretion of glucocorticoids and aberrant function of the (HPA) hypothalamic-pituitary axis, which are common hallmarks of depression, are also subject to GABAergic inhibitory control. Mice with genetically induced $GABA_AR$ deficits in the hippocampus and frontal cortex exhibit impairments in hippocampal neurogenesis (a substrate for some of the antidepressant effects of SSRIs such as fluoxetine)[195] and show chronically elevated HPA axis activity[196]. Indeed, the GABAergic deficit hypothesis of MDD posits that local GABAergic deficits in the hippocampus and frontal cortex (which can arise as a result of

chronic stress) lead to local hyperexcitability, which is then relayed to the paraventricular nucleus of the hypothalamus. There, it promotes excessive release of CRH, triggering increased release of ACTH from the anterior pituitary, which then fosters glucocorticoid release, ultimately creating a positive feedback loop that amplifies cortical and hippocampal GABAergic deficits[197]. Taken together, these many lines of evidence suggest that GABAergic deficits may causally contribute to depressive disorders, while antidepressant therapies may restore normal GABAergic synaptic transmission.

Subtypes of GABAergic interneurons can be defined on the basis of neurochemical, morphological, and/or functional similarity[198]. Three major groups of interneurons—those expressing the Ca2+-binding protein parvalbumin (PV), those expressing the neuropeptide somatostatin (SST), and those expressing the ionotropic serotonin receptor 5HT3a (5HT3aR)—are thought to account for nearly 100% of neocortical GABAergic neurons[199]. Among these, GABAergic cells that co-express SST are perhaps the most interesting interneuron subtype in the context of depression.

SST neurons represent approximately 30% of all cortical inhibitory cells[199,200] and tend to be characterized by dense wiring into the local network and high levels of spontaneous activity that persists in the absence of synaptic input[201]. Many SST-expressing cells exhibit low-threshold regular spiking properties and synapse onto the dendritic tufts of pyramidal neurons to regulate synaptic input integration in pyramidal cells[201]. Intriguingly, evidence from human postmortem and animal studies suggests a selective vulnerability of SST neurons in MDD[202-204]. Work in mice also supports a causal role for reduced SST cell function in mood disorders. SST knockout mice have been shown to exhibit elevated depressive- and anxiety- like behaviors, and disinhibiting interneurons co-expressing SST and GABA has an anxiolytic and

antidepressant-like effect[205]. The neuropeptide SST itself also produces anxiolytic- and antidepressant-like effects when infused into rodent corticolimbic brain regions[206,207].

PV cells are another category of interneurons that have been implicated in the pathophysiology of depression. They compose 30–50% of all inhibitory neurons[199,208] and are typically—but not always—fast spiking cells[209,210] that target the somata and proximal dendrites of both excitatory cells and other PV cells[211] to control spike timing and firing synchrony in principal neurons. Changes in cortical PV expression have been observed in the interneurons of both depressed subjects and animal models of depression, although the evidence here is not as consistent as for SST[170]. In mice, sustained inhibition of PV and SST interneurons has been shown to produce antidepressant-like effects, whereas stimulation of these interneurons prior to drug administration abolishes the antidepressant-like effects of scopolamine[170]. Moreover, mice exposed to an uncontrollable and inescapable stressor exhibited attenuated excitatory synaptic transmission onto PV interneurons specifically in mice showing helplessness, and pharmacogenetic suppression of PV cells in the medial prefronal cortex (mPFC) increased learned helplessness behavior, a model of behavioral despair[212]. Similarly, chronic stress, a crucial vulnerability factor in depression, results in the loss of PV interneurons within the hippocampus[213].

Given that MORs are predominantly expressed in interneurons (and are present specifically on SST- and PV-expressing cells, which are themselves associated with depression), opioid modulation of GABAergic neurotransmission emerges as a possible mechanism through which tianeptine might exert its antidepressant effects. However, the precise role of GABA in depression is still unclear: while the majority of studies suggest that an overall deficit of GABA in the brain results in depressive states (and that antidepressant therapies functionally disinhibit

GABAergic signaling), others find that specific antidepressants such as scopolamine and ketamine may in fact work by inhibiting GABAergic neurotransmission and causing a "glutamate burst". Thus, a simplistic mechanism such as increased or decreased inhibitory tone in the brain is unlikely to explain the nuances of depression and antidepressant efficacy. Specific antidepressant drugs may affect GABAergic signaling differently within distinct brain regions and/or cell types, or have divergent effects based on dose and time course of administration. As such, investigating the putative role of GABA in tianeptine's antidepressant mechanisms will require dissecting these complexities.

1.4.2 Brain Regions of Interest

The mechanism of action for tianeptine can also be investigated through the lens of brain region specificity. Many areas of the brain have been repeatedly implicated in the pathophysiology of depression, and of these, regions that also exhibit a high density of MORs represent prime candidates for tianeptine's site of action in the brain.

1.4.2.1 Hippocampus

The hippocampus has been shown to undergo dramatic changes during depression, including dendritic atrophy, decreased volume, reduced levels of cerebral metabolites, and decreased adult neurogenesis[130,214-216]. Moreover, MDD patients who remit with treatment have been shown to have larger pre-treatment hippocampal volumes[217], whereas those with smaller hippocampal volumes were reportedly more prone to relapse[218]. Strikingly, many of the morphological changes to the hippocampus observed in chronically stressed subjects can be reversed specifically by tianeptine[150,153,219,220]. Chronic stress (intermittent daily

immobilization for 3 weeks or daily injections of 40 mg/kg corticosterone) decreases the number and length of apical dendrites of CA3 pyramidal cells in the hippocampus[153,220]. This reduction in dendritic length and complexity is blocked or reversed by chronic administration of tianeptine, but not by a typical SSRI[152]. In a similar vein, chronic fluoxetine has been shown to reverse the inhibition of hippocampal neurogenesis induced by corticosterone treatment or inescapable stress in mice[221,222]. These neurotrophic effects suggest one possible mechanism in which antidepressants exert their therapeutic effects by reversing neurodegeneration in critical areas of the mood regulating circuit[223,224]. Nevertheless, it is important to keep in mind that there seem to also be neurogenesis-independent mechanisms of antidepressant action, as evidenced by studies showing that it is possible for antidepressants to maintain some of their effects even when neurogenesis was blocked[221,225].

Numerous studies have investigated how disruption of hippocampal function could contribute to several aspects of major depression. Connectivity studies have identified the hippocampus as one of several regions in a network for emotional regulation that is dysregulated in MDD[226]. When various domains of cognitive function are assessed in depressed patients, the most significant impairment is observed in memory measures that are heavily hippocampus-dependent[227]. Moreover, because the hippocampus is a key regulator of the PFC, its dysfunction in major depression could easily contribute to the concentration deficits observed in MDD. Hippocampal afferents are also critical regulators of both the nucleus accumbens (NAc) and the ventral tegmental area (VTA); impairment of this hippocampal function could thus lead to reduced dopaminergic tone and contribute to anhedonia, the loss of pleasure in previously rewarding stimuli[228].

The opioid system likely plays a role in hippocampal plasticity and function, as it is an opioid-rich brain region that expresses all opioid receptors and their associated ligands[229]. Thus, its function may be crucially dysregulated in depression and normalized by antidepressant treatment. The dentate gyrus exhibits high expression levels of all receptors, and MORs and DORs are also expressed in interneuron populations within the hilus and—to a lesser extent—on granule cells[230,231]. Given the high incidence of co-localization between opioid receptor immunoreactivity and interneuron markers, including somatostatin and parvalbumin[230-232], it is unsurprising that the net effect of MOR and DOR activation in the hippocampus is primarily disinhibitory. Activation of MORs or DORs suppresses GABAergic neurotransmission by hyperpolarizing interneurons[233], resulting in a net excitatory effect in the hippocampus[234-236]. In the dentate gyrus, MOR and DOR antagonists impair the induction of long term potentiation (LTP)[236,237], consistent with their known effect of indirect pyramidal cell activation. MORs and DORs have also been shown to modulate activity-dependent synaptic transmission in distinct hippocampal pathways that regulate different aspects of learning and memory, such as contextual associations, memory consolidation, and retrieval[238].

There is some evidence to suggest that mu opioid signaling may be involved in the structural changes to regions such as the PFC and hippocampus observed in depressed patients. In rats, chronic MOR activation using high-doses of morphine (~10 mg/kg) differentially modulates the dendritic arbors of cortical neurons: in the motor or visual cortices, dendritic arborization becomes less complex, whereas in the mPFC, neurons develop longer, more complex dendritic arbors[239-241]. MOR agonists have also been shown to modify dendritic spines, whose morphology is correlated with synaptic plasticity[242]. Notably, this effect appears to be contingent on how an MOR agonist promotes the internalization of its

receptor[243]. Exposure to high-doses of morphine decreases spine density in the hippocampus[244], whereas antagonism of MOR using naltrexone increases spines in the hippocampus, among other regions[245].

In sum, there exists a substantial body of work implicating the hippocampus in depression, and numerous studies elucidating how the opioid system can modulate the hippocampal circuitry underlying a variety of cognitive functions and plasticity mechanisms that are dysregulated in MDD. These two lines of evidence make hippocampus a prime candidate for the site of action of tianeptine.

1.4.2.2 Amygdala

The amygdala is centrally implicated in affective modulation (particularly negative emotions and fear, as well as the emotional aspect of pain), memory encoding, and social behavior[246,247]. As it is a key structure in the limbic-thalamic-cortical network that is supposed to regulate mood, it is unsurprising that the amygdala has also been extensively implicated in depression.

MDD is strongly associated with increases in amygdala size and activity[166]. Several structural imaging studies have reported increased amygdala volume in patients with major depression[215,248,249], and individuals with depression have been found to exhibit increased amygdala activation in response to negatively valenced stimuli[250-253]. In fact, its activity correlates with the intensity of negative affect[254]. Conversely, amygdala response to positive stimuli are often blunted in patients with depression, as evidenced by reduced amygdala activity in depressed individuals passively viewing happy facial expressions compared to control

subjects[255]. Notably, this may be reversible with antidepressant therapy, as successful treatment with citalopram has been shown to increase amygdala responses to happy faces[256].

Animal studies corroborate these findings and highlight a striking dichotomy in hippocampal and amygdaloid responses to stress and depression. In rats, chronic stress has been found to produce opposing patterns of dendritic remodeling in the amygdala and hippocampus, eliciting dendritic atrophy in hippocampal CA3 pyramidal neurons and increasing dendritic arborization and synaptic connectivity in pyramidal neurons of the basolateral amygdala[154,257]. Similarly, while chronic stress impedes hippocampus-dependent declarative learning, it simultaneously enhances amygdala dependent fear learning and anxiety[156,258]. These morphological and functional changes could be the result of and/or contribute to the overactivation of neuronal circuits responsible for modulating fear, anxiety, and emotion.

Moreover, MORs in the amygdala are thought to play a role in controlling anxiety-and depression related responses. MORs are highly expressed in the intercalated nuclei—densely-packed GABAergic neurons interspersed between central amygdala (CeA) and basolateral amygdala (BLA)—and BLA, and modestly so in the CeA[84,259-262]. Locally administering the MOR agonists morphine (at low doses) or DAMGO into the central amygdala (CeA), produced anxiogenic effects in the elevated plus maze[263,264], whereas injecting the selective MOR antagonist CTAP had the opposite effect[264]. Additionally, microinjection of the opioid antagonist naltrexone into the central, but not the basolateral, amygdala blocks the anxiolytic effects of diazepam in the elevated plus maze[265]. It has been proposed that stimulation of MORs in the BLA may diminish nociception and the affective behavior associated with pain by attenuating GABAergic synaptic inputs to CeA-projecting BLA neurons[266]. Finally,

reductions in mu opioid neurotransmission were observed in the amygdala during sustained sadness in humans, significantly more so for MDD patients than for healthy controls[267,268].

1.4.2.3 Habenula

The habenula regulates monoaminergic systems, including dopamine and serotonin, and helps to integrate cognitive, emotional, and sensory processing[269]. It encodes both the rewarding and aversive aspects of external stimuli, and may also be a key player in MDD, as it has been shown to exhibit elevated metabolism across multiple animal models of depression (amphetamine withdrawal, chronic stress, α-methyl-para-tyrosine challenge)[270,271] and to have activity that is strongly correlated with depression severity in human patients[272]. The habenula was one of several brain regions that showed reduced cerebral glucose metabolism following ketamine administration in patients with treatment resistant depression[273], and one post-mortem study discovered significant reductions in cell volumes, numbers, and areas in the medial habenula of depressed individuals[274]. Animal studies have implicated the habenula in a variety of other processes disrupted in depression, including the sleep–wake cycle, antinociception, and behavioral inhibition[269]. Additionally, increased activity in the habenula is associated with enhanced stress sensitivity in a rodent model of learned helplessness[275-277], suggesting that habenular dysfunction may contribute to the etiology of depression by influencing anhedonia and sensitivity to aversive outcomes[269,278].

The habenula can be divided into medial and lateral regions. Most depression-related habenula research that discriminates between these subdivisions has focused on the lateral habenula (LHb), broadly establishing that LHb hyperactivity is associated with depressive-like symptoms, whereas LHb inhibition produces an antidepressant effect [279,280]. However, the

medial habenula (MHb) is more relevant to an examination of tianeptine's effects, as expression of MOR is far denser in the medial compared to the lateral subsection[281]. Direct evidence linking the MHb to MDD is more limited, but MOR knockout has been shown to dysregulate the functional connectivity between the MHb and a number of brain regions involved in reward and aversive behaviors[282]. Moreover, mice lacking MORs in the MHb exhibited diminished conditioned place aversion and withdrawal in response to naloxone, providing yet another link between this region and aversion processing[283]. Additionally, genetic ablation of the dorsal sub-nucleus of the MHb has been found to reduce wheel running activity and sucrose preference, which may be analogous to altered physical activity and anhedonia in depressed human patients[284].

1.4.2.4 Ventral Tegmental Area

Ventral tegmental area (VTA) dopamine neurons project to GABAergic medium spiny neurons (MSNs) of the ventral striatum, which project back to the VTA either directly or indirectly. This pathway is central to the mesolimbic reward circuitry, and may be involved in the anhedonia and motivation deficits observed in most individuals with depression[285]. In animal models of depression, stress has been observed to potently activate VTA dopamine neurons and to stimulate dopaminergic transmission to its limbic targets[286,287]. There are also some reports that antidepressant treatments can alter dopaminergic activity in the VTA or its targets [288].

MORs are densely expressed at both VTA and NAc sites, mostly in GABAergic neurons, and have been implicated in the modulation of dopamine and opiate reward[289,290]. The rewarding nature of VTA MOR activation is clearly demonstrated by the willingness of rodents

to self-administer MOR agonists directly into the VTA[291-294]. Mechanistically, MOR agonists in the VTA have been found to increase both dopamine release in the NAc[295-297] and the firing of putative VTA dopamine neurons[294,298-301]. Furthermore, this MOR agonist-induced increase in NAc dopamine release can be inhibited by co-administering GABA receptor antagonists into the VTA[302]. These results, coupled with the findings that VTA MORs are predominantly expressed on inhibitory interneuron axonal terminals, but not on dopaminergic cell bodies[290,303], and that MOR agonists hyperpolarize these GABAergic interneurons without directly affecting dopaminergic neurons[302,304], suggests a possible circuit for MOR regulation of mesolimbic reward. This canonical two neuron model of opioid reward proposes that MOR excites midbrain VTA dopamine neurons indirectly by hyperpolarizing local GABAergic interneurons[297,305], thereby providing a potential mechanism through which tianeptine could influence mood via modulation of VTA excitability.

1.4.2.5 Striatum

The mammalian striatum, consisting of the dorsal striatum and nucleus accumbens (NAc), receives input from dopaminergic (DA) neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), is a key neuronal substrate for natural and drug rewards[306]. 95% of the striatum is comprised of medium spiny neurons (MSNs), a special type of GABAergic inhibitory cell[307]. MSNs have two primary phenotypes: D1-type MSNs of the "direct pathway" which send inhibitory projections to the VTA and substantia nigra, and D2-type MSNs of the "indirect pathway" which convey information to the VTA indirectly through synapses in the ventral pallidum[308]. MOR is present in the striatal projection neurons of both pathways, although it is more highly expressed in D1 than in D2 cells in the rat striatum[309]. In

mice, selective MOR expression in D1 MSNs restores the rewarding (CPP) and locomotor effects of morphine and partially restores the motivation to self-administer an opiate[310].

Because NAc has a central role in the mechanisms of natural reward, its dysregulation in depression is thought to relate to symptoms of anhedonia[285,311,312]. NAc activity is reduced in major depression, and attenuated NAc activation was found in response to a variety of positive stimuli in depressed subjects[313,314]. Several cases of profoundly refractory depression were successfully treated by deep brain stimulation of the nucleus accumbens[315], and tianeptine in particular has been shown to increase NAc extracellular DA levels as well as DA turnover in rodents[316]. Studies of the MOR distribution in the NAc by autoradiography[259] and light microscopic immunocytochemistry[303,317] have shown patches of intense MOR labeling in GABAergic neurons. Moreover, the shell of the Nac contains a "hedonic hotspot" where microinjections of MOR agonists increase "liking" responses in rodents[318]. This suggests that the NAc is a crucial center for MOR-mediated affective pleasure responses and thus represents another candidate location for tianeptine's site of action.

1.4.2.6 Ventral Pallidum

The ventral pallidum (VP) is a reward-related structure in the basal forebrain that contains largely GABAergic projection neurons and receives substantial GABAergic input from the NAc[319,320]. The VP is characterized by an abundance of enkephalin[321], a mu and delta opioid receptor ligand, and the expression of opioid receptors[322] and mRNA[323]. Enkephalin in the VP arises from the NAc, where it is coexpressed with GABA and D2 dopamine receptors[321]. The colocalization of enkephalin with nonopioid neurotransmitters has also been observed in other brain regions, and it has been suggested that enkephalin inhibits the release of

the co-expressed neurotransmitter[320,324]. Administration of MOR agonists into the VP lowers extracellular GABA levels in the region[325] and reduces the inhibitory effect of NAc projections on VP neurons[326]. These findings suggest that extracellular release of GABA in the VP is strongly influenced by the signaling activity of MORs on presynaptic terminals of afferents from the NAc. Thus, the reward modulating effects of VP MOR activation may be mediated by via the inhibition of GABA neurotransmission.

MOR activation in the VP also appears to have a protective role against depressive states. Mu-opioid neurotransmission in the VP was reduced during sustained sadness, and this was associated with higher negative and lower positive affect ratings by the volunteers involved [267]. By contrast, enhancements in VP mu-opioid neurotransmission correlated with the suppression of the negative affective state elicited by that stressor[327]. Moreover, much like the NAc, the posterior VP contains another "hedonic hotspot" where injections of MOR agonists increase both "liking" and "wanting" of food rewards[319], as well as a hedonic "coldspot" in the anterior VP suppresses "liking" and eating behaviors[328]. Thus, VP MORs are well situated to mediate opiate-based antidepressant responses.

1.4.2.7 Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) connects to both the "emotional" limbic system and the "cognitive" prefrontal cortex and thus likely has an important role in integration of neuronal circuitry for affect regulation. The ACC can be functionally divided into a dorsal cognitive division and a ventral (subgenual) affective division: the former is involved in the cognitive aspects of emotion including conflict resolution of emotional stimuli with negative valence[329-331] while the latter communicates extensively with both limbic regions (e.g. amygdala and

dorsomedial thalamus) and cortical mood regulating areas (e.g. lateral and medial orbitofrontal cortex and the medial prefrontal cortex)[329,330]. Because the ACC is involved in making reward choices, particularly during more complex decisions that require the integration of both cost and benefits[332-334] and plays a critical role in stress and emotional regulation, it has been the subject of extensive study regarding the pathophysiology of depression[335].

Drevets *et al.* used PET imaging to show decreased metabolism in the subgenual cingulate in familial depression[336], and Mayberg and colleagues described abnormalities in both the subgenual and dorsal ACC in depression[337]. Depressed patients and normal subjects experiencing induced sadness or anticipating pain show activation of the affective ventral subdivision and deactivation of the dorsal cognitive subdivision. As such, it is unsurprising that remission has been shown to be characterized by increased activity in the cognitive dorsal region of the ACC[337-339]. Reductions in the metabolic function of the rostral anterior cingulate have also been associated with poorer responses to antidepressant medication in patients with a diagnosis of major depression[340-342]. Moreover, the ACC has also been regarded as a potential DBS site for the treatment of MDD[343].

There is also evidence that this brain region is affected by neuropathological processes that could be associated with abnormal MOR[267,344] and GABA[345] availability. The ACC has been shown to have abnormally low GABA levels in MDD, and depressed patients with low ACC GABA levels have reduced hippocampal volumes compared to healthy controls and to MDD with high GABA levels[346]. Increased mu-opioid activity in the ACC has been observed in response to a prolonged sadness induction paradigm in women with MDD[268]. The activation of mu-opioid neurotransmission in the dorsal ACC has been shown to suppress the affective quality of a sustained pain experience, as evidenced by negative correlations between

pain-specific MPQ (McGill Pain Questionnaire) affective scores and μ-opioid receptor system activation[344]. In a later study, Zubieta *et al*. found that self-induced sustained sadness (from thinking about a sad event) was associated with a significant deactivation in mu-opioid neurotransmission in the rostral ACC. This deactivation was reflected by increases in *in vivo* MOR availability and was correlated with the increases in negative affect ratings and the reductions in positive affect ratings during the sustained sadness state[267].

Thus the ACC represents a convergence between affect regulation, GABAergic signaling and mu-opioid neurotransmission, making it another promising contender for tianeptine's site of action.

Chapter 2: Materials and Methods

2.1 Mice

All mouse protocols were approved by the New York State Psychiatric Institute Institutional Animal Care and Use Committee at Columbia University, and conform to the NIH Guide for the Care and Use of Laboratory Mice. Experiments were designed to minimize the suffering and number of animals used. Animals were group-housed with free access to food and water (except during the novelty suppressed feeding and sucrose preference tests) and maintained on a 12-hour light-dark cycle. Testing was performed during the light period.

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) whereas MOR KO, DOR KO, ß-arrestin 2 (Barr2) KO, and MOR-floxed mice were bred in-house. MOR KO mice (which have disruptions in exon 1 of the *oprm1* gene) and DOR KO mice (in which exon 2 of the *oprd1* gene is deleted) were originally provided by Dr. John Pintar. Barr2 KO mice (Stock No: 011130) were initially purchased from Jackson. MOR-floxed mice, which have exons 2 and 3 of the *oprm1* gene flanked by the LoxP cassette, and chnrb4 Cre mice (RRID:MMRRC_036203-UCD), which express Cre recombinase in neurons with the B4 nicotinic acetylcholine receptor subunit, were provided by Dr. Brigitte Kieffer. Mice expressing one allele of Cre recombinase driven by the VGAT (Stock No: 016962,), SST (Stock No: 013044), PV (Stock No: 008069), or D1 (Stock No: 37156) promoters were also obtained from The Jackson Laboratory. Mice with floxed MOR on both alleles were bred with mice expressing one allele of a given Cre line to produce cell-type specific knockout of MOR. This breeding process involved multiple crosses. First, homozygous floxed mice of interest (Oprm1 fl/fl) were bred to a Cre transgenic mouse strain. Offspring that were heterozygous for the *loxP* allele and hemizygous/heterozygous for the *cre* transgene were then mated back to the homozygous *loxP*-

flanked mice. Offspring from this cross homozygous for *loxP* and hemizygous/heterozygous for *cre* were either used as experimental mice or (if male) maintained for breeding future cohorts.

Genotyping was performed using samples of tail tissue in two independent PCR assays to confirm the presence of floxed MOR on both alleles, and the presence or absence of Cre recombinase. MOR floxed Cre negative littermates were used as controls. MOR and DOR KO mice were bred using heterozygote breeding pairs to obtain MOR KO mice with WT littermate controls.

2.2 Drugs

Tianeptine sodium salt was provided by Servier or purchased from Nyles7.com. The drug's identity and purity were independently verified using NMR spectroscopy. Fluoxetine hydrochloride was purchased from Anawa Trading (Zurich, Switzerland) and morphine sulfate injection, USP from West-ward (Eatontown, NJ). For acute behavioral tests, tianeptine was administered via intraperitoneal (i.p.) injection at a dose of 30 mg/kg, given 15 min (for hot plate) or 1 hour (all other tests) prior to behavioral testing. When assessing tolerance and withdrawal, morphine was administered via subcutaneous (s.c.) injection at 5 mg/kg and naloxone at (1 mg/kg; Sigma Aldrich). The small-molecule mu opioid antagonist cyprodime (10 mg/kg; Tocris Bioscience) was administered by s.c. injection 15 minutes prior to tianeptine administration.

For chronic experiments, corticosterone (CAT #: C2505, Sigma, St Louis, MO) was dissolved in 0.45% hydroxypropyl-β-cyclodextrin (β-CD; CAT #: 297561000, Fisher Scientific, Waltham, MA) at 35 ug/ml. It was delivered in opaque bottles to shield it from light and available *ad libitum* to animals in their drinking water, as described previously[221]. After 4

weeks of corticosterone treatment, mice were also given twice daily i.p. injections of tianeptine (30 mg/kg in 0.9% sterile saline) for another 1-4 weeks (as specified in the figures/legends), after which behavioral testing commenced. For chronic fluoxetine experiments, corticosterone-treated mice were administered 18 mg/kg/day of fluoxetine via oral gavage. 0.9% saline was used as a control for both drugs. All behavioral tests were conducted at least 18 hours after the last drug administration to avoid any acute effects.

2.3 BrdU and DCX Immunohistochemistry

Immunohistochemistry of bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU) and doublecortin (DCX) was used to assess the effects of chronic antidepressant treatment on cell proliferation and survival, respectively. Corticosterone-treated mice were given saline, fluoxetine, or tianeptine for 28 days, injected with BrdU (4 x 75 mg/kg, i.p. dissolved in saline) on the final day of treatment, and then sacrificed 24 hours later. Mice were anesthetized with ketamine and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were post-fixed in 4% PFA at 4°C overnight and transferred into 30% sucrose. Serial sections (40 µm) were sliced coronally through the hippocampus on a cryostat (Leica model CM3050 S) and stored in PBS with 0.1% sodium azide.

The tissue was rinsed with TBS, soaked in 1:1 formamide and 2x SSC (65˚C) for 2 hours, washed in 2x SSC, soaked in 2N HCl (37˚C) for 30 minutes, shaken in 0.1 M boric acid at RT for 10 minutes, and washed again in TBS. Next, sections were quenched in 1% H_2O_2 in a 1:1 PBS:methanol solution for 15 minutes and blocked with 5% NDS in 1x TBS with 0.3% Triton X-100 for 2 hours. Sections were then incubated overnight (4˚C) with primary antibody. After washing with PBS, sections were incubated for 2 hours at room temperature with secondary

antibody. For BrdU staining, these primary and secondary antibodies were rat anti-BrdU (Serotec, 1:100, Cat# OBT0030G) and biotinylated donkey anti-rat (Jackson,1:500, Cat# 702-065-153), respectively. For DCX, we used goat-anti DCX (Santa Cruz, 1:500, Cat# sc-8066) and biotin-conjugated donkey anti-goat (Jackson, 1:500, Cat# 705-065-147). Staining was visualized using DAB. Sections were mounted, dried overnight, dehydrated, cleared with Citrasolve, and then coverslipped with DPX.

Bright-field images of the hippocampus were taken with a Zeiss Axioplan-2 upright microscope. BrdU+ cells were counted manually. Because DCX labeling was so intense in fluoxetine-treated brains, individual cells could not be counted. Instead images were converted to black and white using Otsu thresholding, and the number of black pixels in each image were quantified using ImageJ.

2.4 Chronic Varied Odor Restraint Stress

Chronic varied odor restraint stress (CVORS) pairs the traditional restraint protocol with a randomized series of odors to prevent habituation to the stressor. Mice were horizontally immobilized for 30 min/day within a plastic envelope that was then inserted into a rigid tube. A drop of non-alcoholic odorant was applied to a piece of cotton nestlet and placed by each animal's nose during restraint. Mice were exposed to a random rotation of 6 different odors (strawberry, satsuma, coconut, peppermint, mango, lemongrass) plus a no-odor condition. After being restrained, they were immediately returned to their home cages. Control mice were handled similarly, but not restrained, and were be exposed to the odorant via a drop on a cotton nestlet placed in their cage. For experiments comparing 1 and 4 weeks of tianeptine administration, mice underwent restraint for 3 weeks before starting drug injections. For

experiments investigating the prophylactic potential of tianeptine, stress and drug treatment were started simultaneously.

2.5 Behavioral Testing

Behavioral tests were ordered from least to most stressful: sucrose preference test, open field test (OFT), home cage feeding test, novelty suppressed feeding (NSF) test, forced swim test (FST), and hot plate test. Mice were allowed at least a day to recover in between testing sessions.

2.5.1 Open Field Test

The OFT involves placing a mouse in an open arena and observing its exploratory and locomotor behavior. Mice were put into the OF apparatus (16”x16”x16”) in the dark for 30 minutes, and locomotor behavior was collected and analyzed with MotorMonitor software (Kinder Scientific). For the acute SST and PV cohorts, mice were placed in the OF arena in low light (70 lux) for 20 minutes and tracked using Actimetrics’ Limelight 2 Video Tracking System (Coulbourn Instruments), as the original open field setup had to be relocated. All open field apparatuses were wiped down in between tests so that scents from previous mice would not influence the behavior of subsequently tested mice.

2.5.2 Home Cage Feeding Test

The home cage feeding test measures hunger/feeding behavior (e.g. acute hypophagia in response to opioids). Mice were food-restricted for up to 18 hours and placed into holding cages. Individual mice were then placed back into their home cages, which contained one food pellet of

known weight. After 5 minutes, the mouse was removed and pellet weighed to determine the amount consumed during the testing period.

2.5.3 Novelty Suppressed Feeding Test

The NSF is a task in which food-deprived animals must navigate conflicting desires to remain in the "safe", dark corners of a novel arena or risk venturing into the brightly-lit center to eat. It is sensitive to the effects of both anxiolytics and chronic antidepressants.

Mice were food-restricted for up to 18 hours, then individually placed in the corner of a brightly lit (1200 lux) novel arena (16"x20") which contained a single food pellet affixed to a circular white platform at its center. The time it took for the animal to bite into the pellet was recorded as the latency to eat, and the pellet was immediately removed afterwards. If the mouse did not take a bite of chow within 6 minutes, it was removed from the arena and the latency was recorded as 360 seconds. Following the arena test, mice were returned to their home cage, and their latency to eat in that familiar environment was used as a control measure of hunger drive independent of anxiety-like behavior in a novel arena.

2.5.4 Forced Swim Test

The forced swim test (FST) is a rodent behavioral assay that is commonly used to evaluate antidepressant efficacy, given its well established sensitivity to the actions of classical antidepressants such as SSRIs and TCAs. Mice treated with these drugs display reduced immobility when placed in inescapable containers of water, and this change has been interpreted as an increased motivation to escape and/or reduced behavioral despair.

Two days of FST were conducted as previously described[221]. Mice were placed into clear plastic buckets (20 cm diameter, 23 cm deep, filled with 24°C–26°C water) and videotaped

to assess escape-related mobility behavior. Mice were tested 5 at a time, and opaque, rectangular dividers were placed in between each bucket to prevent animals from seeing each other. Afterwards, mice were gently dried off with paper towels before being returned to their home cage. Only the last 4 minutes of the 6 minute test were scored for mobility duration, as established by Porsolt *et al.* [347]. Scoring was automated using Videotrack software (ViewPoint, France).

2.5.5 Conditioned Place Preference Test

The conditioned place preference (CPP) test was performed using a two-chambered plexiglass Med Associates CPP apparatus. On day 1, mice were allowed to habituate to the apparatus. For 8 subsequent days, mice underwent preference conditioning in which they learned to associate tianeptine or saline with a particular chamber (15cm x 15cm each). Mice were injected with alternating daily injections of tianeptine (30 mg/kg) and saline (control mice received saline every day), and were then placed in one of two chambers of the apparatus (entry into the other chamber is blocked by a plexiglass door) immediately following injection for 45 minutes and allowed to explore undisturbed. On the last day, the mice underwent a 20-minute test session in which they were allowed to freely explore both chambers. Time in each chamber was measured to determine development of a place preference conditioned by drug-induced euphoria.

2.5.6 Hot Plate Test

The hot plate test measures analgesia by observing how long it takes for animals to exhibit signs of discomfort (e.g. licking their hind paws or jumping) after being placed on a heated surface. For this test, mice were placed into a clear glass beaker on the center of a hot

plate. The temperature at the inside edge of the beaker was 50°C. The time it took for the mouse to jump was recorded by an experimenter. If an animal had not jumped after 30s, it was removed from the beaker in order to prevent tissue damage. For the tolerance assays, mice were tested on a hot-plate apparatus set to 55 °C (Bioseb BIO-CHP, Vitrolles, France), using the same 30s cutoff.

2.5.7 Withdrawal Test

Withdrawal-like behavior was assessed 4 hours after the hot-plate test (during which mice had been given either drug or saline). Animals were then subcutaneously administered naloxone (1 mg/kg) or saline and immediately placed into a beaker. Mice were observed for 15 min, and the number of jumps was counted by an observer blind to treatment condition.

2.5.8 Sucrose Preference Test

Sucrose preference tests for anhedonia-like behaviors by observing whether mice prefer to drink water or a palatable sucrose solution. In order to motivate drinking behavior, mice were water-restricted for 8 hours a day for 4 days during the light cycle, and offered a choice of solutions during the dark cycle. After the second day of testing, mice were given one rest day in which they were allowed to drink normally. Testing resumed for two consecutive days afterward.

During the four testing days, mice were provided with two bottles, one with water and the other with 1% sucrose. CVORS mice were not restrained during their testing days, but were restrained during the rest day. The side of the cage that the water and sucrose bottles were presented on were alternated to account for side bias. Fluid consumption was recorded daily and averaged over the four testing days.

2.6 RNAscope

In order to confirm targeted knockdown of MOR expression in the various Cre lines, mRNA in situ hybridization (ISH) was performed on fresh frozen brain tissue using RNAscope© (Advanced Cell Diagnostics, ACD, Hayward, CA) technology. Gene expression was visualized using the RNAscope Fluorescent Multiplex Assay (cat. no. 320850) according to the manufacturer's protocol.

Slides were fixed in prechilled 4% paraformaldehyde in 1x PBS for 15 min at 4˚C and dehydrated in successive 5 minute baths of ethanol (50%, 70%, 100%, 100%) at room temperature (RT). After drying, slides were pretreated with Protease IV for 30 min at RT, washed twice for 3 minutes with 1x PBS, and then hybridized with various commercial probes for MOR (Mm-Oprm1, #315841; Mm-Oprm1-O4-C2, #544731-C2), VGAT (Mm-Slc32a1, #319191), and/or SST (Mm-Sst-C3, #404631-C3) for 2 hours at 40˚C in a humidity chamber. Next, six amplification steps (Amp1-FL, Amp2-FL, Amp3-FL, Amp4-FL-ALT B) were performed at 40˚C in the humidity chamber, each followed by two 2-min washes with 1x RNAscope wash buffer. Finally, slides were incubated with a DAPI counterstain for 30 seconds prior to being coverslipped with Aqua-Mount® Mounting Medium (Thermo Scientific #41799-008).

RNAscope images were acquired using Leica confocal microscopy (405 laser for DAPI, 488 or 552 for MOR, 552 for VGAT, and 638 for SST). 2-3 sections from each mouse were selected for manual quantification, and cells containing more than 5 puncta were considered positive. Five animals were included per genotype.

2.7 [^{3}H]DAMGO Autoradiography

Slides were removed from the -80 freezer and incubated in binding buffer [50mM Tris-HCl,120mM NaCl] for 30 min at 4˚C. They were then placed onto a slide rack (designated for radioactivity) and incubated with a hot buffer solution of 4nM [^{3}H]DAMGO for 1 hour at RT, behind a plastic shield. Slides were then washed twice in binding buffer (for 10 minutes each time), allowed to air dry overnight in the dark, and then exposed to film for 10-12 weeks before developing.

In order to quantify MOR binding in regions of interest (ROIs), developed film was scanned and then analyzed with ImageJ and an Excel macro. Binding density was calculated by background subtraction from a brain region with no detectable binding. A standard curve made using the optical binding values from a set of tritium standards (American Radiolabeled Chemicals, Inc., St. Louis, MO), enabled the extrapolation of binding values of the ROIs. At least four values were averaged for each brain region per mouse.

2.8 Cannulations

Mice underwent stereotaxic surgery in which a bilateral guide cannula was implanted into the ventral hippocampus (Bregma -3.6, ML ±2.8, DV -3.5). The animals were handled daily and habituated to having injectors taken in and out of their guide cannulas. Caps inserted into the cannulae ensured that no particulate matter entered when infusions were not taking place. After one week or more of recovery time, animals were given acute central infusions of either tianeptine or saline, then subjected to FST 15 minutes later. Following FST, mice were placed in an open field arena to assess locomotor effects.

To centrally inject tianeptine, a 26 ga infusion needle was inserted through the surgically implanted guide cannula in each brain hemisphere. The infusion needles were attached by polyethylene (PE50) tubing to 10 µl Hamilton syringes, which were controlled by a microinfusion pump (Harvard Apparatus). The infusion needles extended 200 µm beyond the cannula. A volume of 0.5 µl of the tianeptine solution was delivered at a rate of 0.5 µl/min to each hemisphere. Mice were restrained by scruffing while the infusion needles were being inserted, but not anesthetized.

Chapter 3: Results

3.1 Opioid Mechanisms for Tianeptine's Behavioral Effects

3.1.1 The Acute Behavioral Effects of Tianeptine Require MORs, but not DORs

Given that tianeptine is a full agonist for the mu and delta opioid receptors (with K_is of 383±183 nM and 37.4±11.2 μM for human MOR and DOR, respectively), we sought to determine whether these receptors also mediate tianeptine's behavioral effects. First, we assessed whether transgenic mice lacking MOR and DOR were resistant to the effects of acute tianeptine treatment in a battery of behavioral tests. To do this, we administered either vehicle (0.9% saline) or tianeptine (30 mg/kg) via intraperitoneal (i.p.) injection and then tested behavior 1 hour later.

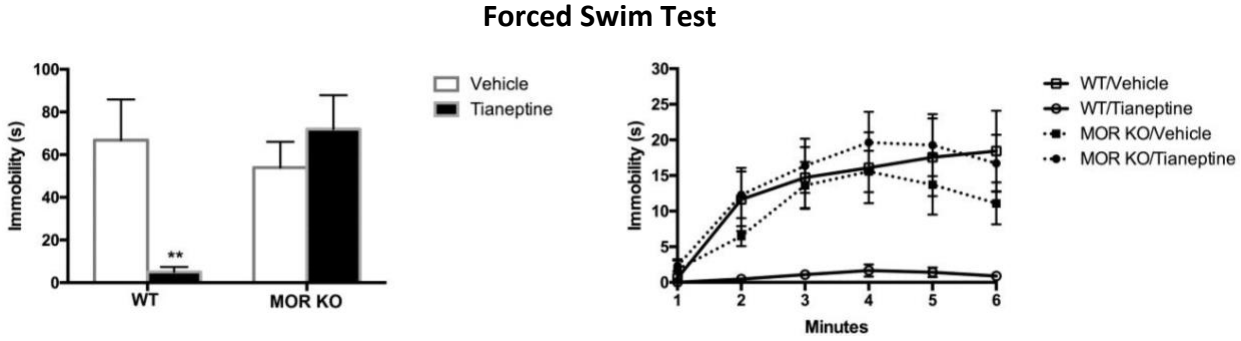

Figure 1: Tianeptine requires MORs for its acute antidepressant-like effects. FST Day 1 Results. Bar graph (left) shows combined immobility results of last four minutes and line graph (right) shows immobility per minute over the six minute test. Immobility duration over the last four minutes was analyzed. Two-Way ANOVA: $F(1,41)=7.876$, genotype x treatment $p=.0076$. ** indicates $p=.0057$ relative to WT/vehicle. n=10-13 per group. Graphs and figure legend were reproduced from [348].

In the FST, a classic predictor of antidepressant efficacy[347], we found that tianeptine decreased immobility compared to saline controls in WT, but not MOR KO mice (Figure 1). Since FST immobility is often interpreted as a measure of "behavioral despair", with longer periods of immobility indicating a depression-like phenotype, these results suggest that tianeptine produces a significant antidepressant-like effect that is lost in mice lacking MOR.

Next, we assessed whether tianeptine produces behavioral phenotypes such as acute hypophagia, analgesia, hyperactivity, and conditioned placed preference, which are commonly observed following administration of morphine and other MOR agonists[349-355]. We found

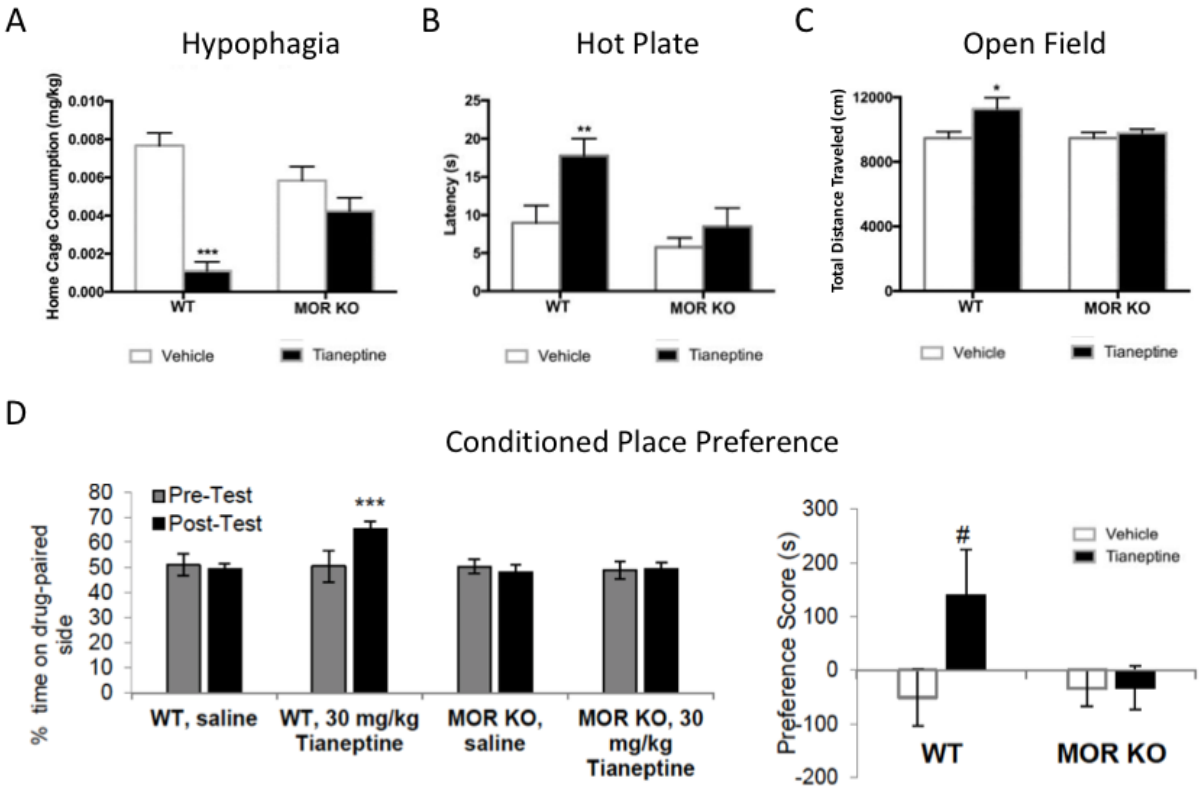

Figure 2: Tianeptine requires MOR for its acute opioid-like effects A) Home cage consumption over 5 minutes after an 18-hour deprivation period was assessed as a measure of hypophagia. Two-Way ANOVA: $F(1,41)=13.72$, genotype x treatment $p=.0006$. *** indicates $p<.0001$ relative to WT/vehicle. B) Latency to jump after being placed on the hot plate was assessed. Two-Way ANOVA: $F(1,40)=1.974$, genotype x treatment $p=.1678$. Planned comparisons: ** indicates $p=.0088$ relative to WT/vehicle. C) Total distance traveled in the open field arena was used as a measure of locomotion effects. Two way ANOVA: $F(1,41)=3.03$, $p=0.09$, genotype by treatment; main effect of genotype: $F(1, 41)=3.03$, $p=0.09$; main effect of treatment: $F(1, 41)=6.32$, $p=0.02$. * indicates $p=0.007$. D) The percent of time spent on the drug-paired side before and after 8 days of context pairings with drug or saline is shown (left). Two-way ANOVA for Pre-test: $F(1, 33)=0.008$, $p>.05$; Two-Way ANOVA for Post-test $F(1,33)=6.99$, $p=0.013$ treatment x genotype. *** indicates $p<.009$ for tianeptine vs saline for WT post-test. The preference score (total time on drug-paired side minus total time on saline-paired side) is shown for the 20 min post-pairing test (right). Two-way ANOVA: $F(1,33)=3.37$, $p=0.075$; planned comparisons: # indicates $p=0.083$. n=10-13 per group. Graphs and figure legend were reproduced from [348].

that, much like morphine, acute tianeptine administration (i.p. injection 1 hr prior to behavioral testing) decreased food consumption in the home cage in WT mice that had been food-restricted for 18 hours (Figure 2A). This hypophagia was notably absent in MOR KO mice (Figure 2A). The analgesic effects of tianeptine were assessed by placing mice on a hot plate and measuring their latency to jump. Again, tianeptine produced a morphine-like effect, increasing the latency to jump off the hot plate 15 min after administration—evidence of an acute analgesic effect—in WT mice alone (Figure 2B). Interestingly, tianeptine did not have a significant analgesic effect one hour after administration, despite producing all other behavioral effects at that time point.

Tianeptine's effects on locomotion were assessed by placing mice into an open field apparatus for 30 minutes, 1 hour after drug administration. As expected for an MOR agonist, tianeptine increased total distance traveled by WT mice in the open field (Figure 2C), but again, this acute hyperlocomotive effect was absent in MOR KO mice. Finally, in the conditioned place preference (CPP) test, which measures associations formed between a rewarding stimulus such as a drug, and a contextual environment (here, two distinct chambers in a CPP apparatus)[356], WT mice showed a markedly increased preference for one chamber after it had been paired with tianeptine, but not saline, suggesting that tianeptine has rewarding properties similar to other opiate drugs (Figure 2D). Once again, the conditioned place preference to tianeptine was absent in MOR-deficient mice (Figure 2D). Overall, these data suggest that tianeptine displays acute antidepressant- and opioid-like properties, both of which require MORs.

The dependence of tianeptine's behavioral effects on MOR was also examined pharmacologically using small-molecule opioid antagonists. We pre-treated mice by injecting them with the MOR-selective antagonist cyprodime (10 mg/kg s.c.)[357] 15 minutes before tianeptine administration, and found that this was sufficient to block tianeptine's antidepressant-

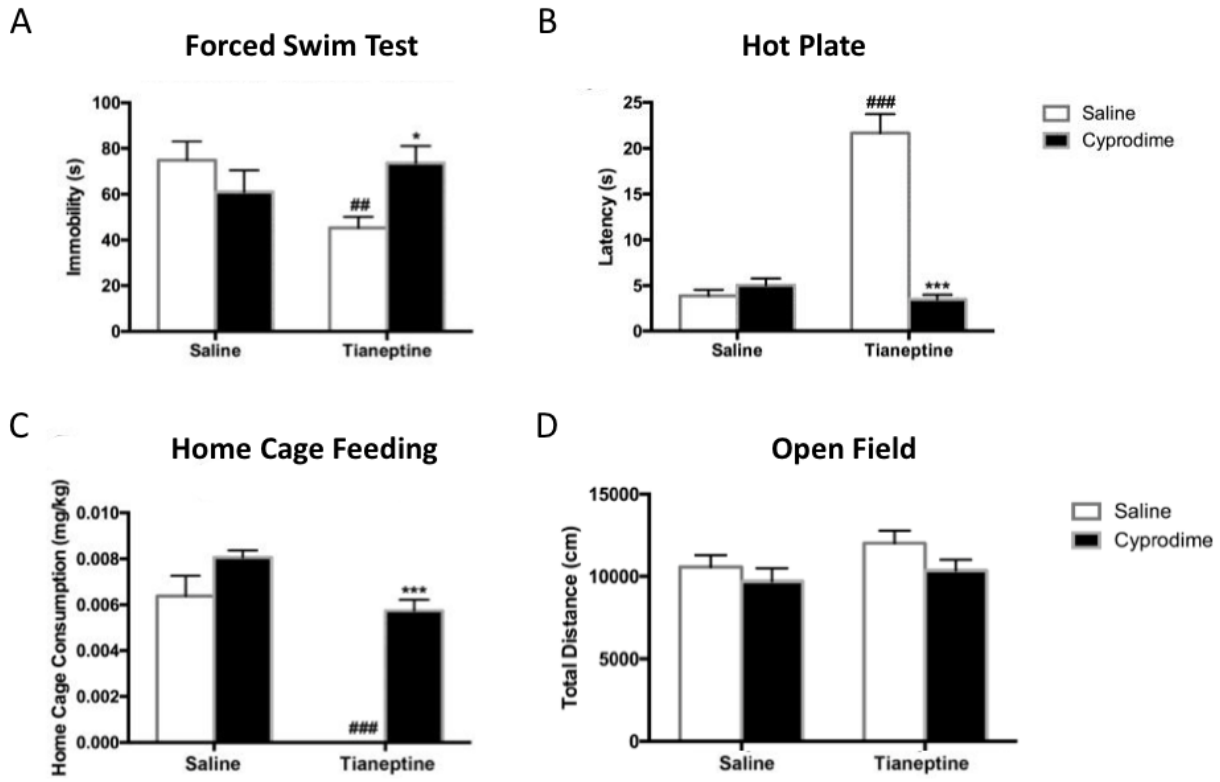

Figure 3: Tianeptine's acute behavioral effects are abolished after pretreatment with a selective mu opioid antagonist. Mice were pretreated with saline (N=9-12) or cyprodime (10 mg/kg sc, N=9). Tianeptine (30 mg/kg) was administered by i.p. injection. A) Bar graph shows combined immobility results of last four minutes in the FST. FST Day 2 results for pretreatment with saline or cyprodime. Two-Way ANOVA: $F(1,35)=7.487$, pretreatment x treatment $p=.0097$. ## indicates $p=.0093$ relative to saline/saline, * indicates $p=.0147$ relative to saline/tianeptine (Fisher's). B) Latency to jump after being placed on the hot plate was assessed. Two-Way ANOVA: $F(1,35)=65.27$, pretreatment x treatment $p<.0001$. ### indicates $p<.0001$ relative to saline/saline, *** indicates $p<.0001$ relative to saline/tianeptine. C) Home cage consumption over 5 minutes after an 18-hour deprivation period was assessed as a measure of hypophagia. Two-Way ANOVA: $F(1,35)=14.90$, pretreatment x treatment $p=.0005$. ### indicates $p<.0001$ relative to saline/saline, *** indicates $p<.0001$ relative to saline/tianeptine. D) Total distance traveled in the open field was assessed as a measure of overall locomotion. Two-Way ANOVA: $F(1, 35)=.29$, $p=0.5$. All bar graphs indicate mean±SEM. Graphs and figure legend were reproduced from [348].

like effects of in the FST, and its opioid-like behavioral effects in the home cage, hot plate, and open-field tests (Figure 3A-D). These results, together with the data from the genetic loss-of-function experiments, confirm that all of our observed behavioral effects of tianeptine depend on MORs.

Thus far, we had only considered necessity of MOR for tianeptine's antidepressant-like effects, but tianeptine is also an agonist at DOR. However, upon performing a battery of

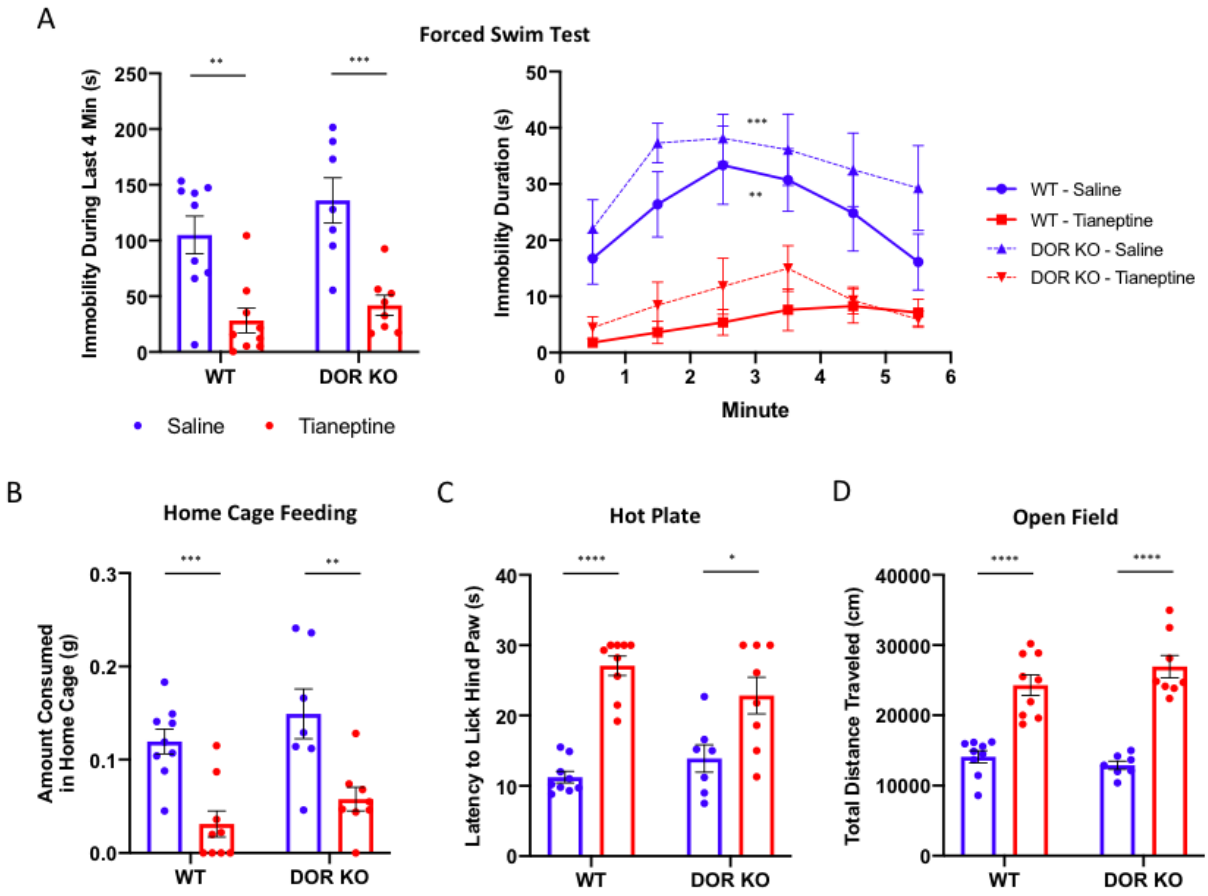

Figure 4: Tianeptine does not require DORs for its acute behavioral effects. A) FST results. (Left) Bar graph shows combined immobility results of last four minutes. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: **p=0.002 for WT; ***p<0.001 for DOR KO (unpaired t-test). (Right) Line graphs show immobility per minute over the 6-minute test. Planned comparisons, saline vs. tianeptine: **p=0.001 for WT and ***p<0.001 for DOR KO (repeated measures two-way ANOVA). B) Home cage feeding over 5 min after an 18-h deprivation period was assessed as a measure of hypophagia. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: ****p<0.001 for WT, **p=0.01 for DOR KO (unpaired t-test). C) Analgesia was assessed using latency to jump after being placed on the hot plate (15 min post-injection). Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: ****p<0.000001 for WT, **p=0.02 for DOR KO (unpaired t-test). D) Open field hyperlocomotion results. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine:**** p<0.0001 for WT, ****p<0.00001 for DOR KO. N=7-9 mice per group. All acute behavioral assays except hot plate were conducted 1 hour after an acute i.p. injection of tianeptine (30 mg/kg).

behavioral tests (FST, home cage feeding, hot plate, open field) on DOR KO mice and their WT littermates, we found that tianeptine continued to have acute antidepressant- and opioid-like effects in every test regardless of genotype (Figure 4A-D), suggesting that MOR, but not DOR are required for tianeptine's acute behavioral effects.

3.1.2 The Chronic Antidepressant-like Effects of Tianeptine Require MORs

3.1.2.1 Rationale for Acute vs. Chronic Testing

The acute and chronic effects of an antidepressant drug are not necessarily the same, nor need they be mediated by the same brain regions or mechanisms. SSRIs are pharmacologically effective after acute administration, but therapeutic improvements are not apparent until several weeks into treatment[8]. Indeed, the initial effects of antidepressant drugs are sometimes even the opposite of their chronic effects[358-360]. So far we have clearly demonstrated that tianeptine has acute antidepressant-like effects in mice, but chronic antidepressant-like effects are more relevant to the study of human depression and response to antidepressants, and must be investigated separately.

Nevertheless, acute testing remains an important step in understanding antidepressant mechanisms, even though these drugs are generally administered chronically. Tests for acute drug effects provide the practical benefit of being fast, while also offering good predictive value, as evidenced by the success of the acute FST in screening for efficacious serotonergic antidepressants. It should be noted that both tianeptine and ketamine produce effects in the FST, despite ostensibly working through non-monoaminergic mechanisms.

Acute tests are also an important step in establishing chronic mechanisms. For one, they enable us to distinguish whether an observed effect in a chronic test is from the most recent

(acute) drug dose of the chronic paradigm or legitimately a chronic effect. In our studies of chronic tianeptine, we addressed this issue by 1) carrying out all behavioral tests at a time point past acute effects and 2) controlling for behaviors observed in acute tests such as hypophagia and hyperactivity, which are potential confounds in chronic tests that involve feeding or locomotive behaviors.

3.1.2.2 Tianeptine Time Course Experiments

In order to determine how long a single dose of tianeptine continues to produce acute behavioral effects, we conducted time course studies assessing the effect of tianeptine at time points 1 hour, 3 hours, and 24 hours post injection. Tianeptine significantly reduced immobility in the FST 1 hour after injection (the time at which all acute behavioral tests except for hot plate were previously conducted), but had no discernable antidepressant-like effect at either 3 or 24

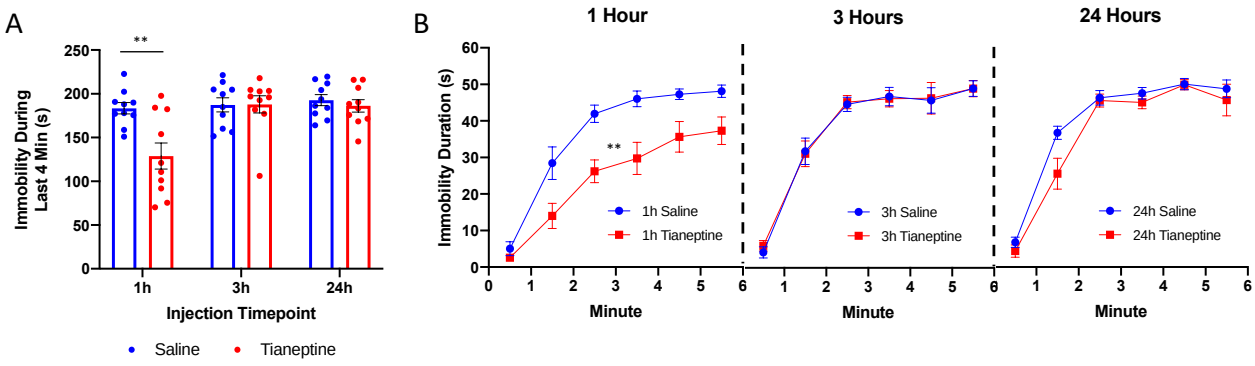

Figure 5: Tianeptine produces acute antidepressant-like effects 1 hour, but not 3 or 24 hours, post-injection. A) Bar graph shows FST immobility over the last 4 minutes. Two-way ANOVA: timepoint x treatment interaction: p=0.0083. Planned comparison, saline vs. tianeptine: **p=0.0036 at 1 hour. B) Line graphs show immobility per minute over the 6-minute test. Planned comparisons, saline vs. tianeptine: **p=0.0049 at one hour (repeated measures two-way ANOVA). N=10 mice per group.

hours after drug administration (Figure 5A-B). Similarly, tianeptine dramatically increased total distance traveled in the open field apparatus at the 1 hour, but not the 3 hour or 24 hour time

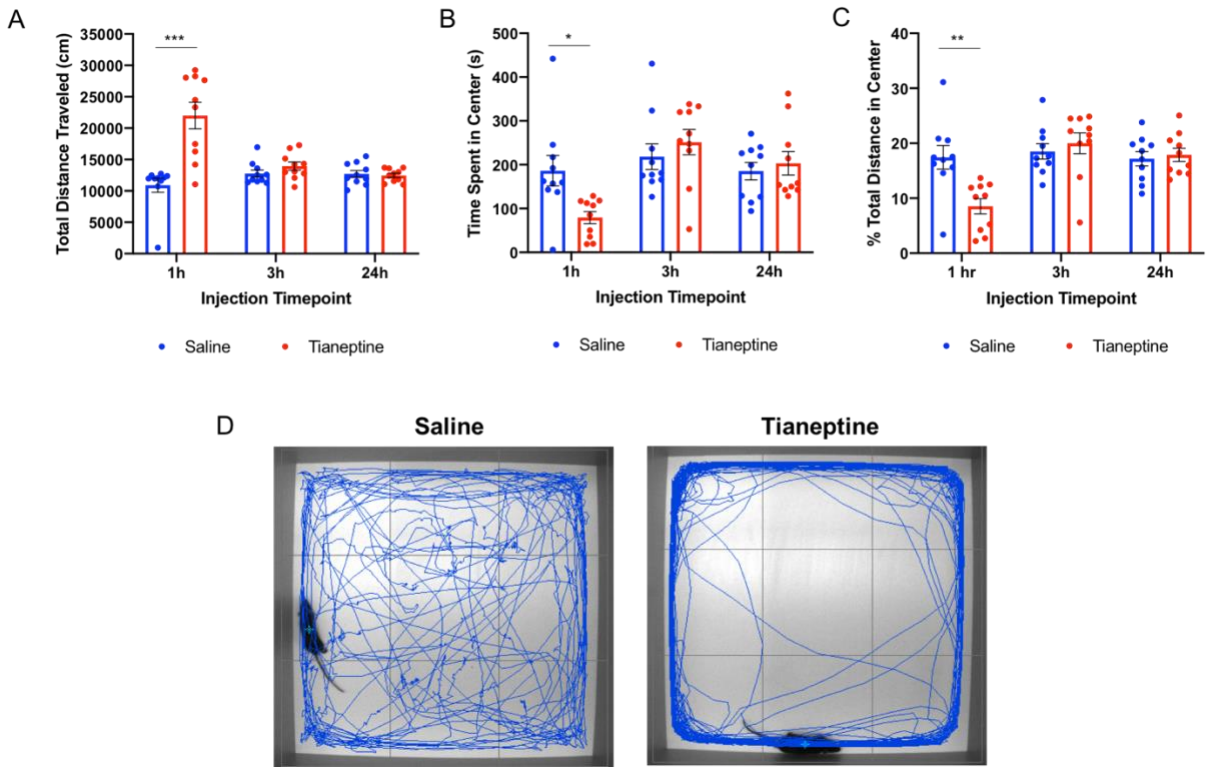

Figure 6: Tianeptine affects locomotor behavior at 1 hour, but not 3 or 24 hours post-injection. A) Bar graph shows total distance traveled in the open field apparatus during a 30 minute test. Two-way ANOVA: timepoint x treatment interaction: $p<0.0001$. Planned comparison, saline vs. tianeptine: ***$p=0.0002$ at 1 hour. B) Time spent in center of the open field. Two-way ANOVA: timepoint x treatment interaction: $p=0.0198$. Planned comparison, saline vs. tianeptine: *$p=0.0104$ at 1 hour. C) Percent total distance traveled in center (calculated as distance traveled in center/total distance traveled). Two-way ANOVA: timepoint x treatment interaction: $p=0.0028$. Planned comparison, saline vs. tianeptine: **$p=0.0028$. D) Representative images of locomotor behavior for saline (left) and tianeptine (right) treated mice, 1 hour post injection. All bar graphs indicate mean±SEM. N=10 per group.

points, suggesting that tianeptine's opioid-like hyperlocomotive effects also do not persist for long (Figure 6A).

Time spent or percent of the total distance traveled in the center of the open field has sometimes been used as a measure of anxiety-like behaviors, based on the assumption that less anxious animals would be more willing to explore exposed, potentially threatening areas. Although tianeptine has been reported to have anxiolytic effects in both rodent models and

depressed patients[166], and thus should be expected to increase center time and activity, we observed the opposite: tianeptine significantly decreased both the total time spent (Figure 6B), and the percent of total distance traveled (Figure 6C) in the center of the open field 1 hour post injection. However, when we examined the locomotor patterns of tianeptine- and saline-treated mice, we found that tianeptine mice exhibit a stereotyped rapid, circling behavior in which they run laps around the perimeter of the arena, whereas control mice show more normal exploratory patterns (Figure 6D). This suggests that the decreased center time and distance observed in tianeptine-treated mice are artifacts of tianeptine's acute hyperlocomotor effects, rather than indications of increased anxiety in these animals. This confound definitively precludes the use of open field center occupancy as a measure of tianeptine's acute anxiolytic-like effects in future studies. Once again, no behavioral effects were observed at the 3 hour and 24 hour timepoints (Figure 6B-C).

The time-course of tianeptine's antidepressant-like effects were also assessed using the novelty suppressed feeding (NSF) test, an assay that is sensitive to both acute benzodiazepines and chronic antidepressants. In this test, food-restricted mice are placed in a brightly lit arena containing a single food pellet affixed to a platform at its center. Animals must then navigate their conflicting desires to remain in the "safe", dark corners of the novel arena or risk venturing into the brightly-lit, exposed center in order to eat. Following the arena test, baseline hunger was assessed in a home cage feeding test and locomotion was measured in an open field to ensure that any differences in latency to feed were truly a measure of antidepressant/anxiolytic-like effect.

In the NSF test, mice given tianeptine showed increased latency to feed in the novel arena, but again, only at the 1 hour timepoint (Figure 7A-B). At first glance, this too, may appear

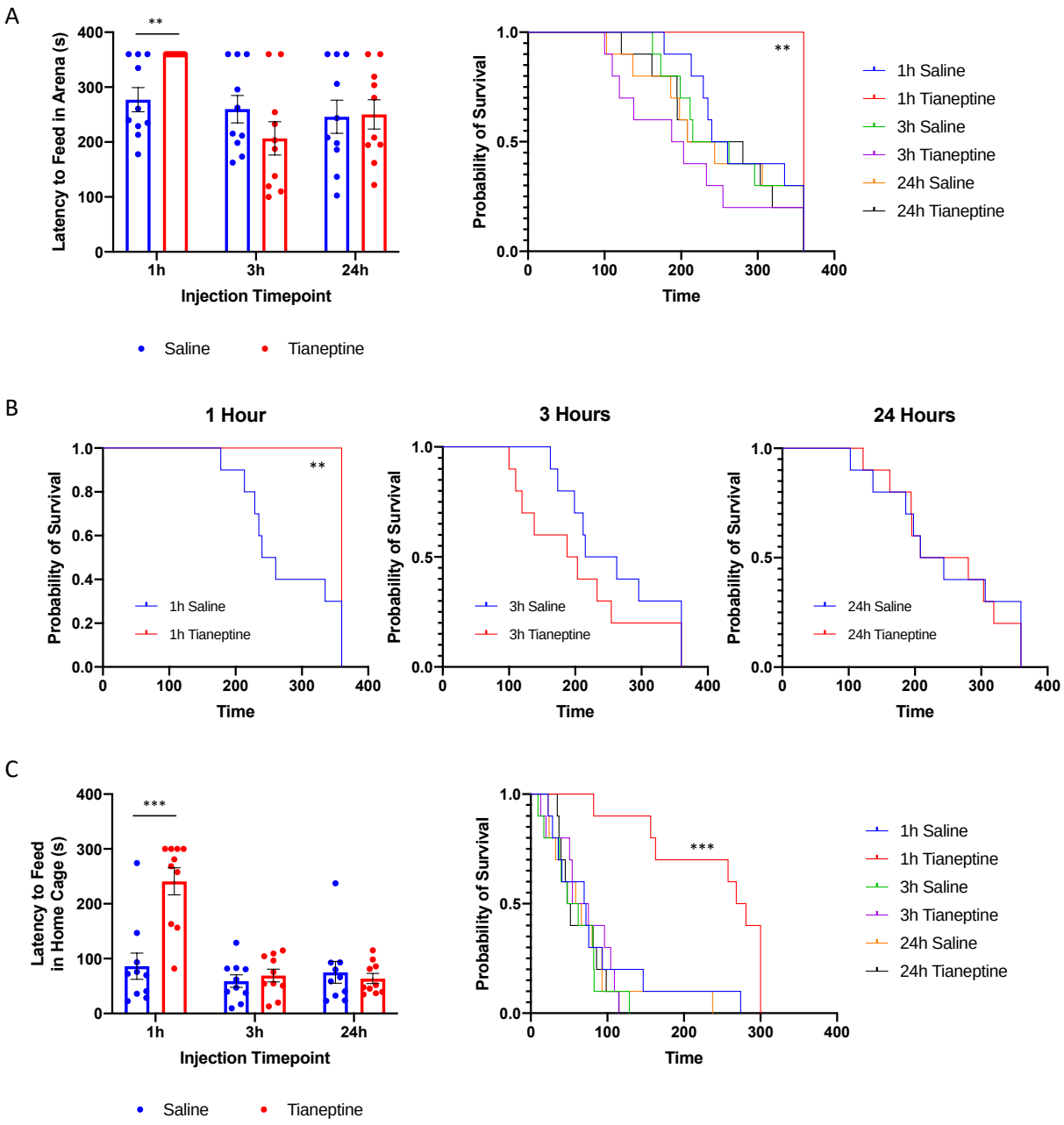

Figure 7: Tianeptine has acute hypophagic effects at 1 hour, but not 3 or 24 hours post-injection. A) Bar graph shows latency to feed in an anxiogenic novel arena at each time point. Two-way ANOVA: timepoint x treatment interaction: p=0.280. Planned comparison, saline vs. tianeptine: **p=0.0014 at 1 hour. B) Survival curves for latency to feed in the arena. Logrank (Mantel-Cox): **p=.0072. C) Survival curves from (B) separated by timepoint. Planned comparison, saline vs tianeptine: *p=0.0011 at 1 hour, p=0.2986 at 3 hours (Logrank, Mantel-Cox). C) Latency to feed in the home cage expressed as a bar graph (left) and survival curve (right). (Left) Two-way ANOVA: timepoint x treatment interaction: p<0.0001. Post hoc t-test, saline vs. tianeptine: ***p=0.0003 at 1 hour. (Right) Logrank (Mantel-Cox): p=.0001. Planned comparison, saline vs tianeptine: ***p=0.0004 at 1 hour. All bar graphs indicate mean±SEM. N=10 per group.

counterintuitive, as tianeptine would be expected to alleviate anxiety- and depression-like states, thereby decreasing latency to feed in the brightly-lit, novel arena. However, when we looked at the home cage control test, we found that latency to feed in a familiar environment was also dramatically increased in tianeptine mice alone, suggesting that the arena results were driven by tianeptine's acute opioid-like hypophagic effects (Figure 7C). This marked suppression of hunger completely disappears by 3 hours after drug administration, as evidenced by uniformly low feeding latencies in the home cage at the 3 and 24 hour timepoints (Figure 7C). Notably, at 3 hours post injection, tianeptine appears to slightly decrease latency to feed in the novel arena (Figure 7B, center). Although this difference is not significant ($p=0.299$), it is a reversal from the results at 1 hour post-injection, when tianeptine increases arena feeding latencies. This could indicate that tianeptine's acute anxiolytic-/antidepressant-like effects are completely masked by an overwhelming blunting of hunger at the 1 hour timepoint, but that residual therapeutic effects may become evident by 3 hours, when hypophagia is no longer a countervailing factor.

When taken together, these results suggest that tianeptine produces acute antidepressant and opioid-like behavioral effects 1 hour post injection, and that these effects largely vanish by 3 hours. This is consistent with published data indicating that tianeptine is rapidly metabolized; in healthy human volunteers, tianeptine reaches its maximum plasma concentration 0.94 hours after a single oral dose, and has an elimination half-life of 2.5 hours[10]. In mice, a single i.p administration of tianeptine is almost entirely eliminated from both plasma and brain tissue after 1 hour, and becomes undetectable in the brain after 2 hours[348]. Tianeptine's MC5 metabolite, however, has a much longer half-life and can be detected in brain tissue for at least 8 hours[348]. As MC5 has been shown to have the same acute antidepressant- and opioid-like effects as

tianeptine[348], we took a safe approach and conducted all chronic behavioral tests at least 16-18 hours after the last acute injection.

3.1.2.3 Chronic Behavioral Testing

In order to assess the behavioral effects of chronic tianeptine administration, we first induced a depressive-like phenotype in mice by exposing them to chronic glucocorticoids.

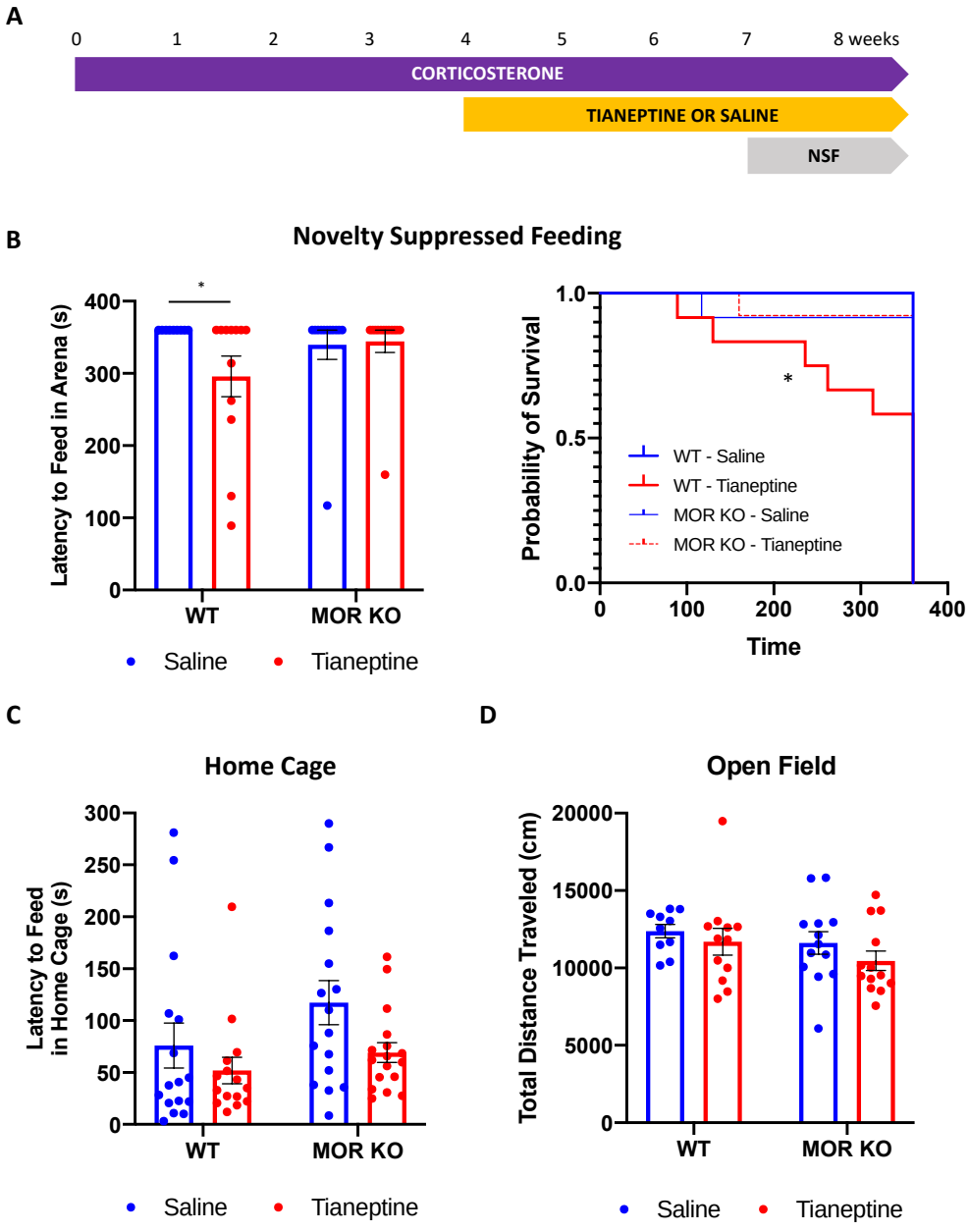

Figure 8: Tianeptine requires MOR for its chronic antidepressant-like effects. A) Timeline for (B-C). Mice underwent 28 days of corticosterone administration via their drinking water (35 ug/ml), followed by twice daily i.p. injections of tianeptine (30 mg/kg) or saline for 21 days before behavioral testing. N=8-10 per group. B) Latency to feed in the novel arena. Logrank (Mantel–Cox Survival): p=0.0262. Planned comparison, saline vs tianeptine: *p=0.033 for WT; p=0.930 for MOR KO. C) Latency to feed in the home cage shows no significant effect of chronic tianeptine on hunger. Logrank (Mantel–Cox): p=0.2202. D) Total distance traveled in the open-field arena measured 18 hours post tianeptine injection shows no significant effect of chronic tianeptine on locomotion. Two-way ANOVA: treatment × genotype interaction: p=0.74. Bar graphs indicate mean±SEM. Data was originally published in [348].

Following 28 days of corticosterone administration via the drinking water, mice were given twice daily i.p. injections of tianeptine (30 mg/kg) or saline for 21 days (Figure 8A). The chronic antidepressant/anxiolytic-like effects of tianeptine were then assessed using the NSF test. Crucially, this behavioral assay has been shown to be sensitive to chronic, but not acute treatment with fluoxetine, suggesting that it mimics the clinical presentation of SSRIs.

We found that chronic tianeptine reduced latency to feed in WT mice compared to saline treated controls, but did not have an effect in MOR KO mice (Figure 8B). By contrast, neither feeding behavior in the home cage (Figure 8C), nor total distance traveled in the open field (Figure 8D), were affected in either genotype, indicating that the observed NSF effects were not directly confounded by any residual hunger or hyperactivity effects of acute tianeptine. These results demonstrate that chronic tianeptine treatment produces antidepressant-like effects in an MOR-dependent fashion.

We next sought to determine whether chronic tianeptine could ameliorate stress-induced depression-like behavior in a different behavioral paradigm. Chronic corticosterone is a convenient method of inducing depressive-like states in rodents, but it is also a very artificial manipulation, and there is some evidence to suggest that it may be ineffective in females[361]. Thus, we also decided to use the chronic varied odor restraint stress (CVORS) model for

depression. CVORS pairs traditional chronic restraint stress with various odors in order to prevent animals from habituating to the stressor. It is more naturalistic than chronic corticosterone in that mice actually experience chronic stress rather than having their glucocorticoid levels pharmacologically manipulated. Experimental mice are immobilized in

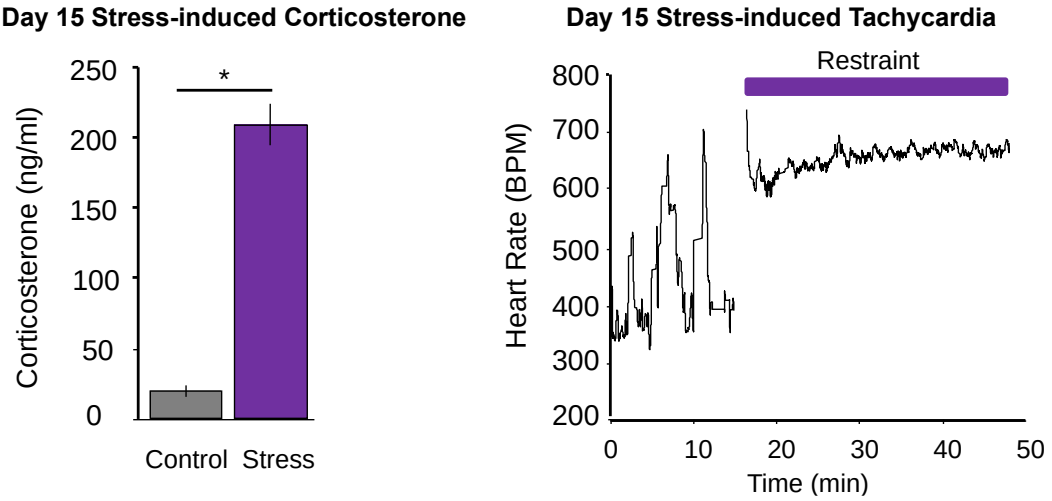

Figure 9: CVORS remains stressful over time. Left) Bar graph shows blood corticosterone levels after 15 days of CVORS. $p<0.05$ by unpaired rank-sum test. N=12,16. Right) Heart rate before and during restraint for a representative mouse during day 15 of CVORS. Graphs reproduced with permission from Alexander Harris.

decapicone bags placed into conical tubes for 30 minutes a day, during which they are presented one of 7 odor conditions in a pseudo-random order via a drop of odorant placed in the bags. Alexander Harris has found that even after 15 days of CVORs, restraint continued to cause both elevated blood corticosterone levels (Figure 9A) and stress-induced tachycardia (Figure 9B), indicating that mice did not become acclimated to the stressor, even after repeated exposure to it (personal communications).

Crucially, Harris also showed that this paradigm causes hedonic-like deficits (personal communication), which are important symptoms of depression. Specifically, CVORS elicited social avoidance in the social interaction test, which can be interpreted as increased "social

anxiety" (Figure 10A), and decreased preference for a 1% sucrose solution in the sucrose preference test, suggesting that the mice may be experiencing anhedonia-like symptoms (Figure 10B).

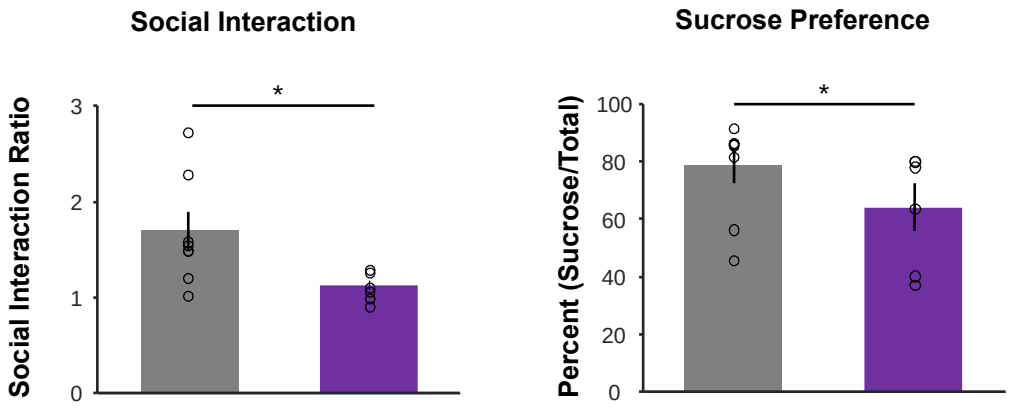

Figure 10: CVORS reduces hedonic-like behaviors. Left) Bar graph shows the social interaction ratio (time spent in the interaction zone in the presence of another mouse/time spent there in the absence of a social target) following CVORS. $p<0.05$ by unpaired rank-sum test. N=7,6. Right) Bar graph shows sucrose preference (volume of 1% sucrose solution consumed/total volume of liquid imbibed) after CVORS. *$p<0.05$ by unpaired rank-sum test. N=6,5. Graphs reproduced with permission from Alexander Harris.

Having established that CVORS produces ongoing stress that develops into a depressive-like state, we then sought to determine whether tianeptine would be effective in counteracting this phenotype, especially in the NSF test, our main assay of chronic antidepressant efficacy. Moreover, we wondered whether tianeptine could be effective in preventing the onset of depression-like behaviors, in addition to alleviating symptoms of an established depressive-like phenotype, as we had shown previously (Figure 8).

To address these questions, we began drug treatment concurrently with CVORS, rather than after. This concomitant stress and treatment and lasted for three weeks, after which we tested the effects of chronic tianeptine in the NSF test (Figure 11A). As one might expect, we found that mice given tianeptine had significantly lower latencies to feed in the arena compared

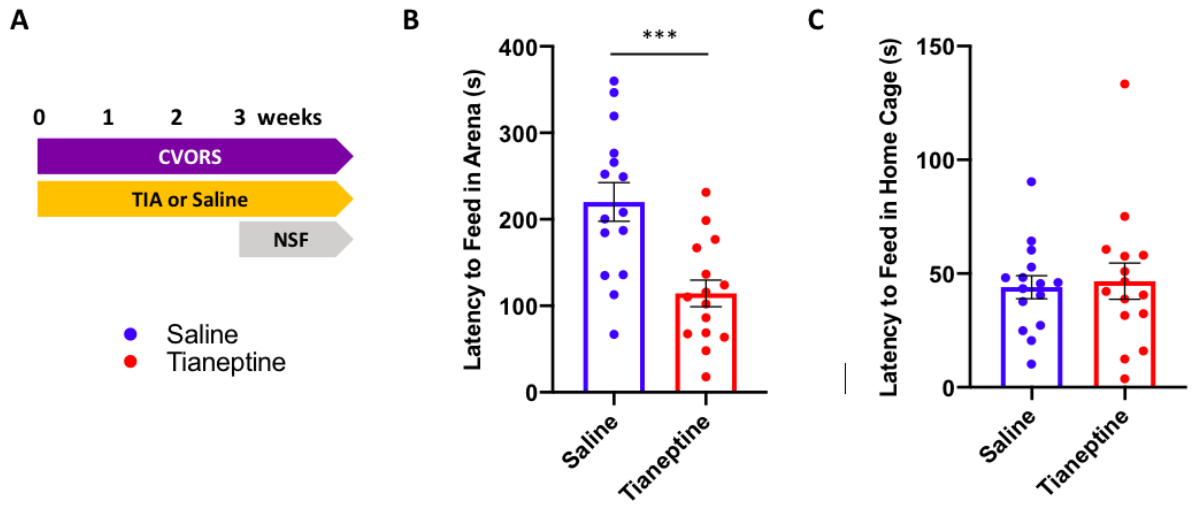

Figure 11: Chronic tianeptine produces antidepressant-like effects in CVORS mice. A) Timeline for (B-C). WT and MOR-KO mice were restrained for 30 min daily in the presence of different odors (CVORS) and injected with tianeptine (30 mg/kg, i.p.) twice daily for 3 weeks. N=15 per group. B) Latency to feed in the novel arena. ***p=.0005 by unpaired t-test. C) Latency to feed in the home cage. p=0.7813 by unpaired t-test. Bar graphs indicate mean±SEM.

to controls given saline (Figure 11B). Moreover, this difference was only present in the novel enclosure, and not in the home cage, suggesting that these results were not confounded by hunger (Figure 11C).

The logical next step was to address whether the observed antidepressant-like effects of tianeptine within this paradigm also required the presence of MORs. To that end, we subjected WT and MOR KO mice to the CVORS paradigm with contemporaneous drug treatment, and assessed their behavior four weeks later in the NSF and in the sucrose preference test, one of the tests on which the CVORS paradigm was originally validated (Figure 10B).

We found that for CVORS-treated animals, WT mice injected with tianeptine had markedly higher sucrose preference scores compared to controls given saline; by contrast, tianeptine treatment did not rescue the sucrose preference in MOR KO mice subjected to CVORS (Figure 12A). Similarly, in the NSF test, tianeptine tended to decrease the latency to

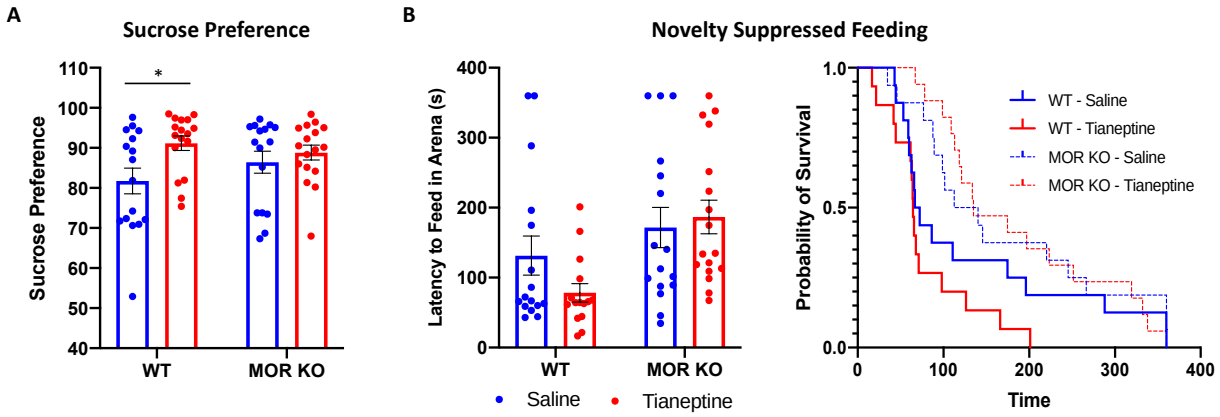

Figure 12: Chronic tianeptine treatment produces MOR-dependent antidepressant-like effects in CVORS mice. A) Sucrose preference (sucrose intake/total fluid intake x 100, averaged over 4 days) was assessed as a measure of anhedonia. Planned comparisons, saline vs tianeptine: *p=0.014 for WT; p=0.470 for MOR KO (unpaired t-test). B) Bar graph (left) and survival curve (right) of NSF arena results. Logrank (Mantel-Cox Survival): p=0.001. Planned comparisons, saline vs tianeptine: p=0.138 for WT and p=0.937 for MOR KO. All behavioral assays were conducted at least 18 h post injection. N=15-17. Bar graphs indicate mean±SEM.

feed in the novel arena for WT, but not MOR KO mice, although this effect was not significant at the 0.05 alpha cutoff (Figure 12B). These results suggest that chronic tianeptine treatment may have somewhat protective effects against the development of anhedonia- and anxiety-like behaviors, and that these effects require the presence of MORs.

3.2 Comparison with Fluoxetine

3.2.1 Tianeptine has a Distinct Mechanism of Action from Fluoxetine

Because tianeptine is an MOR agonist rather than a serotonin modulator, we hypothesized that its mechanism of action would differ to some degree from that of SSRIs like fluoxetine. One such dimension would be the necessity of MORs. To assess whether MORs are required for the antidepressant efficacy of SSRIs, we measured the behavioral response to

chronic fluoxetine in corticosterone-treated MOR KO and WT mice in the NSF test (See Figure 8A for equivalent timeline). In contrast to tianeptine, which loses its antidepressant-like efficacy in MOR KO mice (Figure 8B), chronic fluoxetine treatment decreased latency to feed in the novel arena regardless of genotype (Figure 13A), indicating that MOR expression is not required for the chronic antidepressant-like effects of fluoxetine. It should be noted, however, that fluoxetine tended to decrease latency to feed even in a familiar environment (Figure 13B).

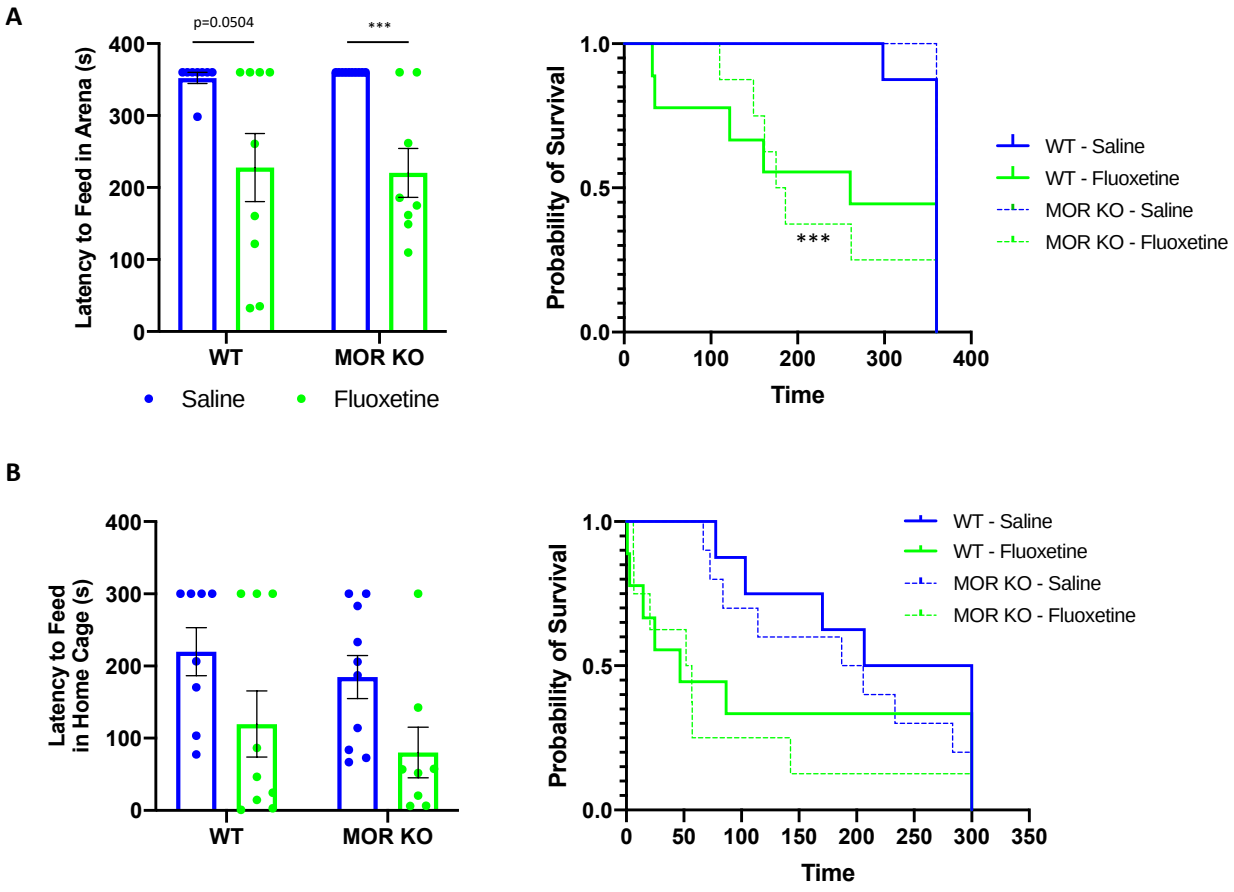

Figure 13: Fluoxetine does not require MORs for its chronic antidepressant-like effects in the NSF. A) NSF results. Latency to feed in the novel arena following 3 weeks of fluoxetine treatment (18 mg/kg/day, oral gavage) in chronic corticosterone-treated mice. Logrank (Mantel–Cox Survival): p=0.001. Planned comparisons, saline vs tianeptine: p=0.050 for WT; ***p=0.001 for MOR KO. B) Latency to feed in the home cage was measured following the arena test. Logrank (Mantel–Cox Survival): p=0.097. Planned comparisons, saline vs tianeptine: p=0.195 for WT; p=0.058 for MOR KO. N=7-10. Bar graphs indicate mean±SEM.

Although this difference was not statistically significant, it still somewhat confounds interpretation of the NSF result by introducing hunger, rather than attenuated anxiety- and depression-like states, as a possible driver for decreased feeding latencies in fluoxetine-treated mice. As such, the behavioral effects of chronic fluoxetine were also assessed using the FST, which robustly detects antidepressant-like effects for SSRIs. Here again, we observe that fluoxetine continues to be effective, even in MOR-deficient mice, as evidenced by lowered immobility times in both genotypes (Figure 14A-B).

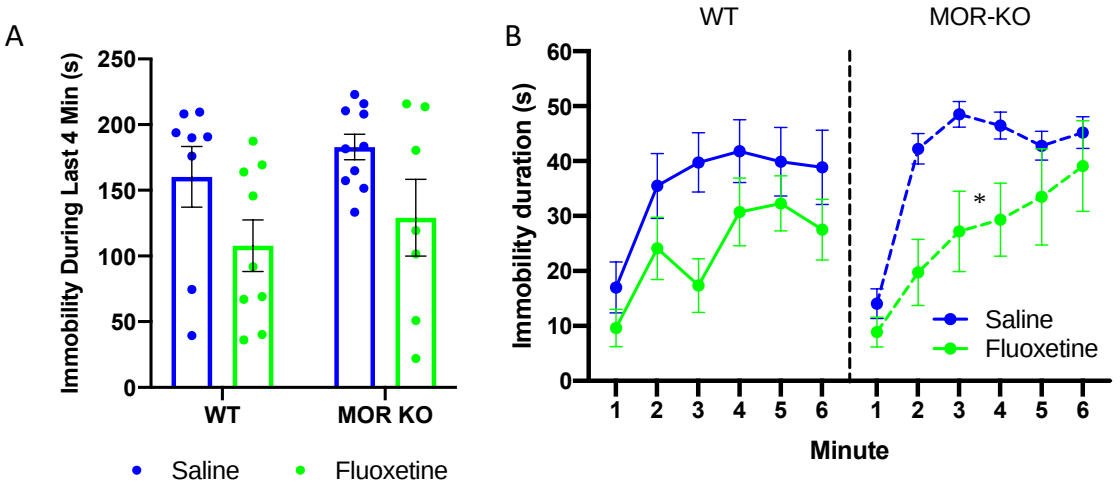

Figure 14: Fluoxetine does not require MORs for its chronic antidepressant-like effects in the FST. A) Bar graph shows combined immobility results during the last 4 minutes of forced swim in corticosterone-treated mice. Two-way ANOVA: main effect of treatment: p=0.0133. Planned comparisons, saline vs. tianeptine: p=0.1026 for WT; p=0.0625 for MOR KO (unpaired t-test). B) Line graphs show immobility per minute over the entire 6-minute test. Planned comparisons, saline vs. tianeptine: p=0.0791 for WT and *p=0.0269 for MOR KO (repeated measures two-way ANOVA).

Intriguingly, while fluoxetine remained effective in MOR KO mice, work by Marley Kass and Elena Carazo showed that it no longer reduced forced swim immobility in DOR KO mice (Figure 15A-B). Taken together with our previous data assessing tianeptine's effects in MOR- and DOR-deficient mice, we see that tianeptine requires MOR, but not DOR for its

antidepressant-like effects, whereas fluoxetine is the opposite, requiring DOR but not MOR. It therefore appears that tianeptine and fluoxetine engage entirely different components the opioid system, suggesting distinct underlying mechanisms of action.

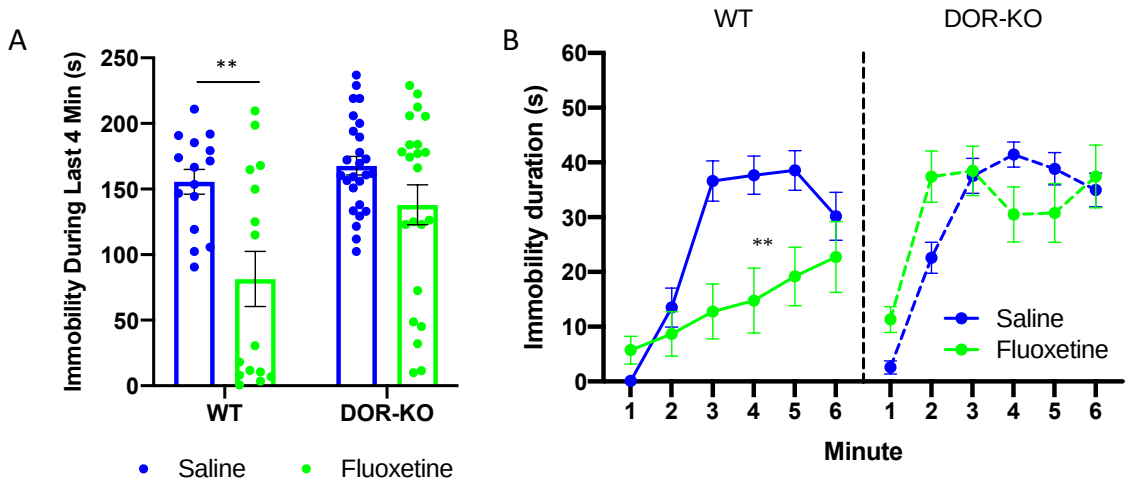

Figure 15: Fluoxetine requires DORs for its chronic antidepressant-like effects in the FST. A) Bar graph shows combined immobility results of the last 4 minutes. Two-way ANOVA: main effect of treatment: p=0.0003; main effect of genotype: p=0.0133; no interaction: p=0.1062. Planned comparisons, saline vs. tianeptine: p=0.0032 for WT; p=0.0676 for MOR KO (unpaired t-test). B) Line graphs show immobility per minute over the 6-minute test. Planned comparisons, saline vs. tianeptine: **p<0.01 for WT and p=0.7417 for MOR KO (repeated measures two-way ANOVA). Figure reproduced with permission from Elena Carazo.

Given that increased hippocampal neurogenesis following chronic fluoxetine treatment contributes to some of its antidepressant-like effects[221], we also examined the effect of tianeptine on cell proliferation (BrdU) and maturation (DCX). We observed small but significant increases in the number of BrdU+ cells following tianeptine treatment, though these were much smaller than those seen following fluoxetine (Figure 16A-B). Unlike the almost fourfold increase in DCX staining following fluoxetine treatment, there was no effect of tianeptine on DCX expression (Figure 16A,C). Thus, tianeptine's effect on brain and behavior differs from that of fluoxetine in at least two important aspects: 1) it requires MORs while fluoxetine does not, and 2) it is likely hippocampal neurogenesis-independent.

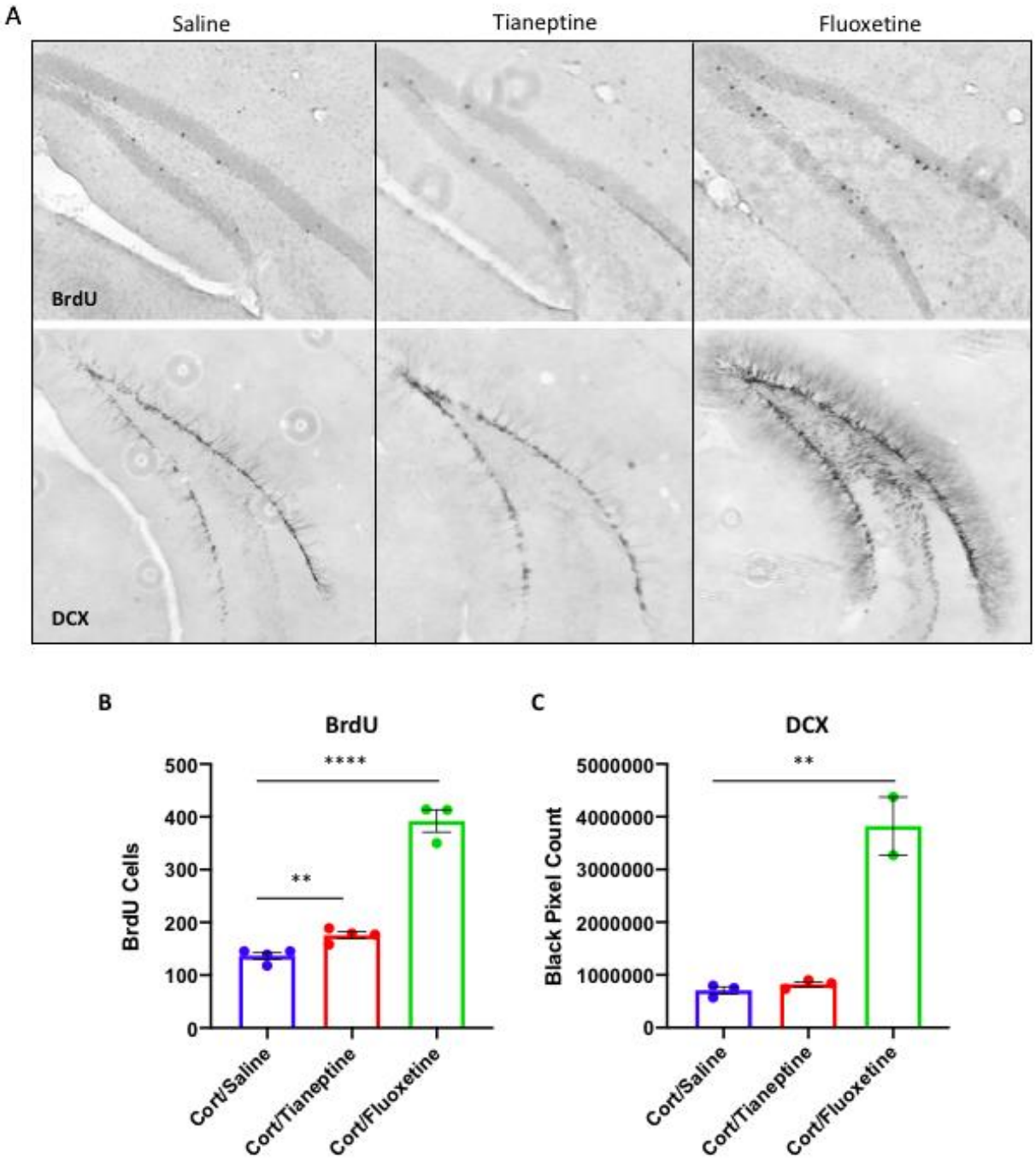

Figure 16: Tianeptine does not promote hippocampal neurogenesis. A) Neurogenesis in the dentate gyrus of the hippocampus was assessed by staining for BrdU (top) and DCX (bottom) following chronic antidepressant treatment. Mice were treated with 0.9% saline (left), 30 mg/kg tianeptine (middle), or 18 mg/kg fluoxetine (right) for 4 weeks, injected with BrdU (4 × 75 mg/kg) on the final day of treatment, and sacrificed 24 h later. B) BrdU positive cells were counted in the dentate (both sides) for every 6th section of the hippocampus. n=3-4 mice per group. One-way ANOVA: $p<0.0001$. **$p=0.005$, tianeptine relative to saline; ****$p<0.0001$, fluoxetine relative to

saline (unpaired t-test). C) Doublecortin expression was quantified by thresholding each image (Otsu) and determining the number of black pixels (DCX stain) in the dentate (both sides) for every sixth section of the hippocampus. n=2-3 mice per group. One-way ANOVA: $p<0.001$. **$p=0.005$, fluoxetine relative to saline (unpaired t-test).

Finally, we sought to determine whether tianeptine requires serotonin for its anti-depressant-like effects, as SSRIs do. Not only does the monoamine hypothesis propose that the actions of monoaminergic drugs are based on modulating serotonin availability at the synapse, animal experiments have directly established that depleting serotonin can abolish fluoxetine's antidepressant-like effects. Para-chlorophenylalanine (PCPA) is a selective and irreversible inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin.

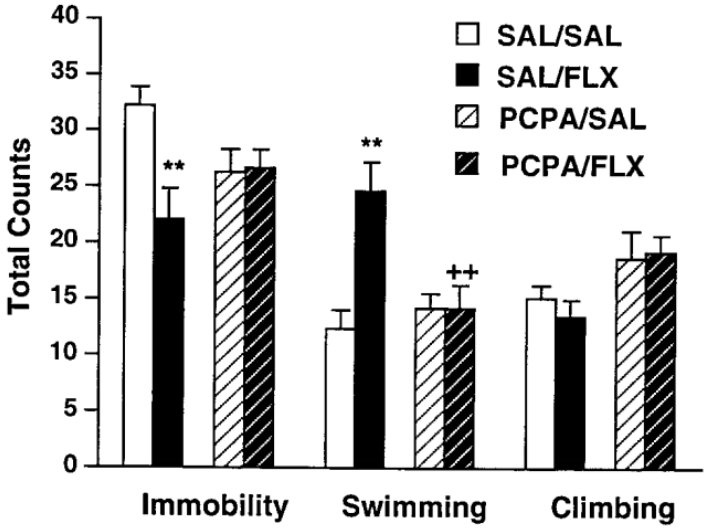

Figure 17: Blockade of the effects of the selective serotonin reuptake inhibitor fluoxetine in the rat FST by pretreatment with PCPA. Mean counts (+1SEM) of immobility, swimming and climbing behaviors are shown when sampled every 5 s during the 5-min FST test period. n=10 rats per group. Rats were treated with saline (SAL, solid bars) or PCPA (hatched bars, 150 mg/kg twice) 72 and 48 h prior to the FST test period to deplete 5-HT prior to behavioral testing. After administration of the pretest, rats were injected with either SAL or fluoxetine (FLX, 20 mg/kg) 23.5, 5 and 1 h prior to the FST test period. Asterisks represent values following fluoxetine that differ significantly from the corresponding saline control group, **P<0.01. Crosses represent corresponding values that differ according to saline or PCPA pretreatment, ++P<0.01. Graph and legend reproduced from [362].

Page *et al*. [362] showed that pretreatment with PCPA eliminates the effect of fluoxetine in the FST: PCPA pretreated mice no longer showed reduced immobility or increased swimming behavior in response to the drug (Figure 17).

We performed a similar experiment with tianeptine, administering a pretreatment of PCPA 150mg/kg twice per day for 3 days in order to deplete serotonin levels prior to behavioral testing. Unlike fluoxetine, however, tianeptine elicited an acute antidepressant-like effect regardless of whether the mice had had PCPA pretreatment, suggesting that tianeptine does not directly engage the serotonin system the way SSRIs do (Figure 18A). Moreover, tianeptine also produced acute hyperlocomotion in the OFT irrespective of pretreatment conditions, precluding the possibility that the FST immobility results were affected by any PCPA-induced locomotor differences (Figure 18B). In order to confirm that PCPA pretreatment had indeed reduced serotonin levels in the brain, we sent the brains of experimental and control mice (2 per group) to John Mann's lab for high performance liquid chromatography (HPLC) of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA).

The HPLC results show a nearly significant reduction in 5-HT ($p=0.0668$) and a modest decrease in its 5-HIAA, suggesting that serotonin was—to at least some degree—depleted, by PCPA pretreatment (Figure 18C). Further work remains to be done, including repeating this experiment with a drug that more effectively depletes serotonin and adding a direct fluoxetine comparison, before definitive conclusions can be drawn. Even as is, however, these results are at least consistent with the rest of our data suggesting that fluoxetine and tianeptine engage distinct molecular mechanisms to produce their antidepressant-like effects.

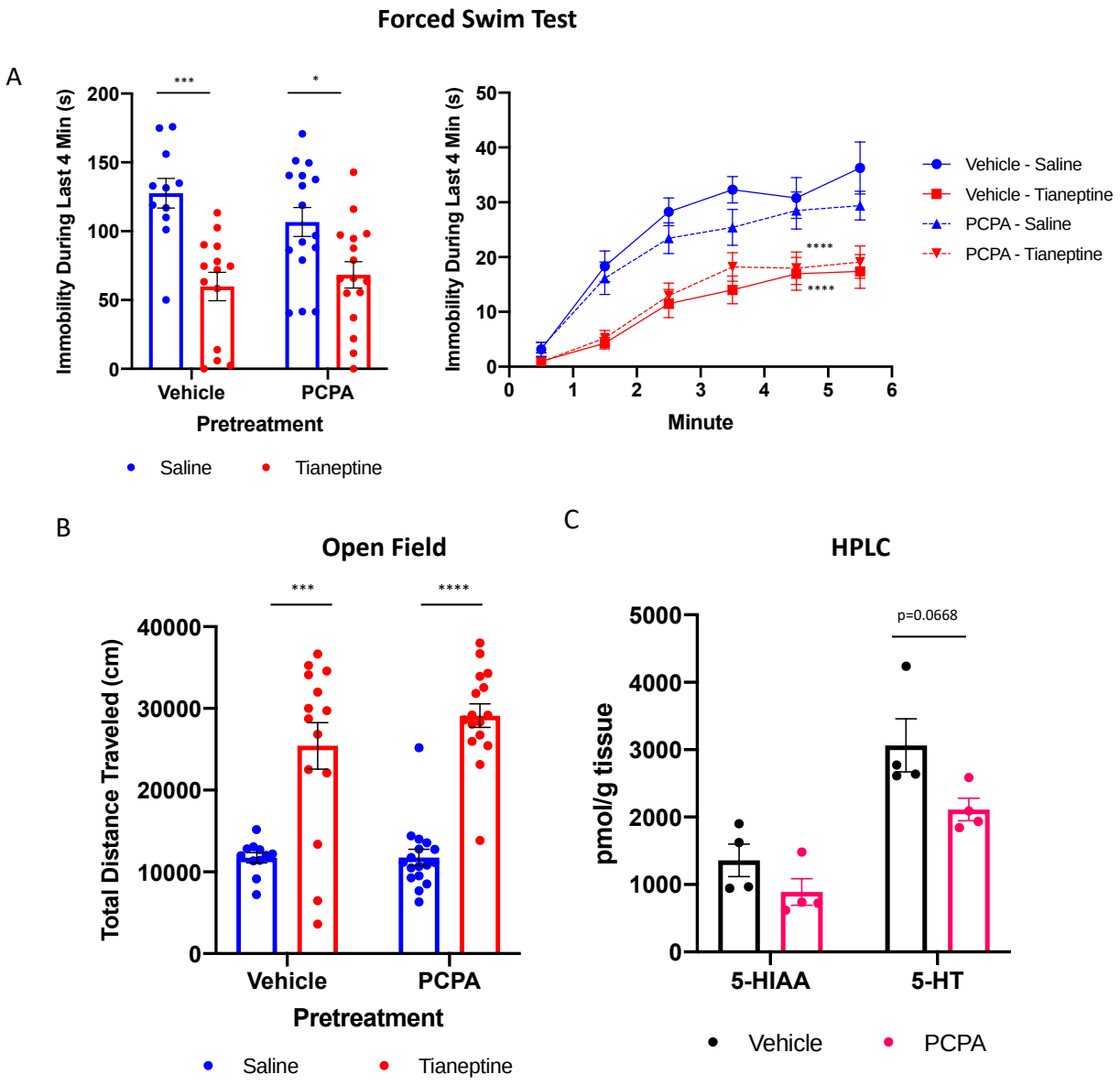

Figure 18: PCPA does not block the acute antidepressant-like effects of tianeptine. A) (Left) Bar graph shows immobility results during the last 4 minutes of the FST. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: ***p=0.0002 for Vehicle; *p=0.0113 for PCPA (unpaired t-test). (Right) Line graphs show immobility per minute over the 6-minute test. Two-way repeated measures ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: ****p<0.0001 for vehicle and ****p<0.0001 for PCPA (repeated measures two-way ANOVA). B) Locomotion was assayed in the OFT following FST. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: ***p=0.0004 for Vehicle; ****p<0.00001 for PCPA (unpaired t-test). C) High performance liquid chromatography (HPLC) was used to measure the extent of serotonin depletion. Two-way ANOVA: main effect of serotonin measure: p<0.0001, main effect of PCPA: p=0.0197. Planned comparisons, vehicle vs PCPA: p=0.1838 for 5-HIAA and p=0.0668 for 5-HT.

3.2.2 Tianeptine Shows Rapid Antidepressant-like action

Another dimension which might distinguish tianeptine from fluoxetine is the time course for its chronic effects. We have established that tianeptine has long-term antidepressant-like effects, and we now wanted to determine whether these effects could emerge after a shorter treatment duration than is required for SSRIs. Once again, we turned to the CVORS paradigm. Following 3 weeks of stress, tianeptine treatment began for 1-4 weeks, followed by NSF (Figure 19A,D). Chronic tianeptine administration significantly reduced latency to feed in the novel

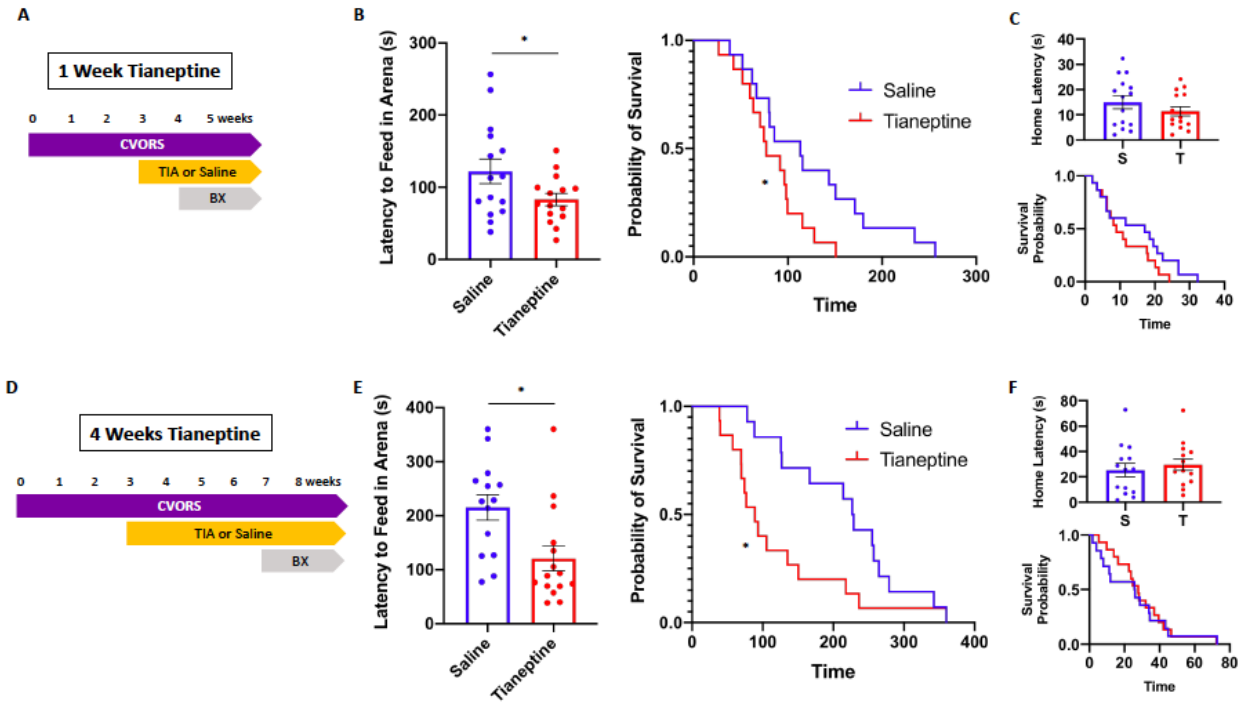

Figure 19: Tianeptine has rapid antidepressant-like effects. A) Timeline for (B-C). n=15 per group. 30 mg/kg tianeptine was administered by i.p. injection twice daily for 1 week following 3 weeks of daily 30-minute restraint and odor exposure according to the CVORS paradigm. B) NSF results. Latency to feed in the novel arena is expressed both as a bar graph (left) and survival curves (right). Logrank (Mantel-Cox Survival): *p=0.042. C) A control measure of latency to feed in the home cage was measured following the arena test. D) Timeline for (E-F). n=14-15 per group. 30 mg/kg tianeptine was administered by i.p. injection twice daily for 4 weeks following 3 weeks of CVORS. E) NSF results. Logrank (Mantel-Cox Survival): *p=0.029. F) Latency to feed in the home cage was measured following the arena test.

arena following both 1 (Figure 19B) and 4 weeks (Figure 19E) of treatment. These results indicate a faster onset of antidepressant-like efficacy than would be expected from SSRI treatment, although there was a smaller effect size following one week compared to four weeks of treatment. Notably, the Javitch lab observed similar results—with tianeptine producing a faster effect than fluoxetine—using the chronic corticosterone paradigm (personal communications). Home cage feeding was not influenced by tianeptine treatment at either timepoint (Fig. 19C, F), indicating that the arena results were not due to tianeptine's effects on hunger.

3.3 Comparison with Morphine

3.3.1 Tianeptine does not Produce the Same Tolerance or Withdrawal Effects as Morphine

Another compound with which tianeptine can readily be compared is morphine: both are MOR agonists, and acutely produce opioid-related phenotypes such as analgesia and reward. However, the dimension that is most interesting and most relevant for tianeptine's utility as an antidepressant drug is abuse liability.

Despite their efficacy in treating pain and possibly depression, the long term use of opioid drugs as treatments is severely limited by their high potential for abuse. Chronic morphine administration, for instance, produces profound tolerance and physical dependence, so we wanted to determine whether tianeptine also elicits these negative effects. Tolerance (in which subjects develop reduced responsiveness to a drug over time, and thus require escalating doses to experience the same therapeutic effect) was assessed by measuring the effect in the hot plate test of acute drug treatment following chronic exposure to saline, tianeptine (30 mg/kg twice daily for 34 days), or morphine (5 mg/kg twice daily, for 10 days). As expected, acute administration

of morphine following chronic administration produced no significant analgesic response in latency to jump in the hot-plate test (Figure 20A). Astonishingly, no such tolerance was observed toward tianeptine. Following chronic administration of tianeptine (30 mg/kg twice daily for over 30 days), acute administration of tianeptine produced a robust analgesic response, which was not significantly different from mice treated chronically with saline (Figure 20A).

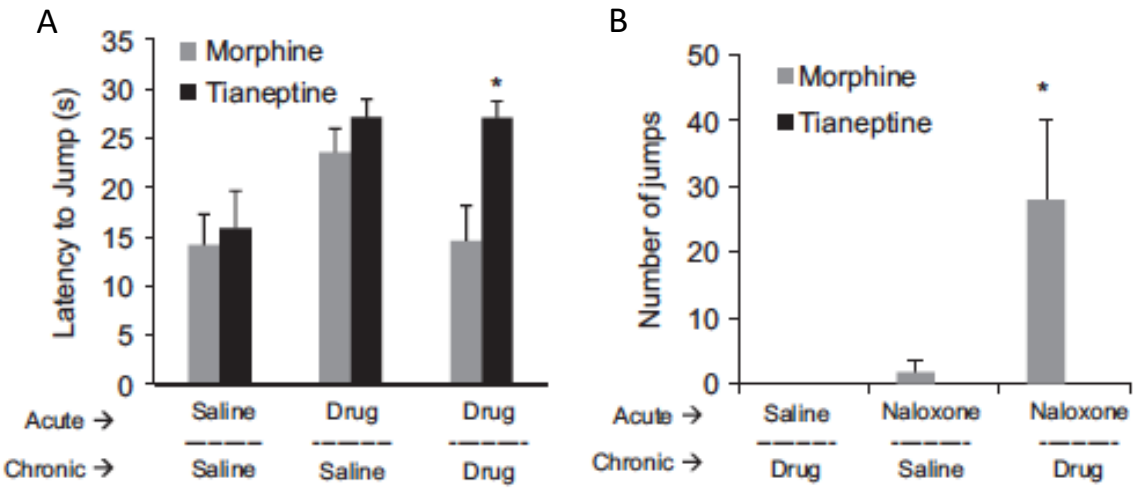

Figure 20: Unlike morphine, tianeptine does not produce tolerance or withdrawal. A) Tolerance was assessed by measuring the effect of acute drug treatment (saline, morphine at 5 mg/kg, or tianeptine at 30 mg/kg) using the hot-plate test following chronic exposure to saline, tianeptine (30 mg/kg twice daily for 34 days), or morphine (5 mg/kg twice daily, for 10 days). B) Withdrawal was assessed through jumping behavior following acute administration of naloxone (1 mg/kg) following chronic exposure to saline, tianeptine (30 mg/kg twice daily for 34 days), or morphine (5 mg/kg twice daily, for 10 days). Two-way ANOVA: $F(2, 47)=10.87$, $p<0.001$ for drug × naloxone treatment. *$p<0.05$ compared to morphine-saline and tianeptine-naloxone. All bar graphs indicate mean±SEM. Graphs and figure legend were reproduced from [348].

Next we assessed withdrawal, in which subjects that have become dependent on opioids experience adverse physical and mental symptoms after stopping or reducing drug intake. Mice treated chronically with morphine (5 mg/kg twice daily, for 10 days) displayed the expected jumping behavior indicative of withdrawal following administration of naloxone (1 mg/kg), a competitive opioid receptor antagonist (Figure 20B). However, mice chronically treated with

tianeptine (30 mg/kg twice daily for 34 days), did not display this jumping behavior after naloxone administration (Figure 20B). Thus, while tianeptine may have rewarding effects similar to those of morphine, it does not appear to induce the same level of tolerance or withdrawal.

3.3.2 Tianeptine's Acute Behavioral Effects do not Require ß-arrestin 2

The finding that tianeptine, despite being a full MOR agonist, does not produce the same tolerance and withdrawal that morphine does, could potentially be explained by a difference in biased GPCR agonism between the two: morphine is known to mainly recruit the G protein, rather than the ß-arrestin signaling pathway, but this is not necessarily the case for tianeptine. Moreover, ß-arrestin 2 has been shown to play a role in mediating responsiveness to the mood stabilizer lithium[363], and clinical studies have also implicated ß-arrestins in MDD and stress[364,365], suggesting that this pathway may be broadly important in the context of depression. Work from the Javitch lab suggests that tianeptine engages both G protein and ß-arrestin signaling, but the specific contributions of each pathway to tianeptine's antidepressant- and opioid-like effects remains unknown (personal communications).

Thus, in order to investigate the possible role of ß-arrestin 2 signaling in mediating the behavioral effects of tianeptine, we assessed the effects of acute tianeptine treatment in ß-arrestin 2 (Barr2) KO mice. Tianeptine continued to reduce immobility in Barr2 KO mice, indicating that its antidepressant-like effects are not dependent on the presence of ß-arrestin 2 (Figure 21A). Similarly, in the open field, home cage feeding, and hot plate tests, tianeptine produced hyperlocomotion, hypophagia, and analgesia respectively (Figure 21B-D), regardless of genotype, suggesting that the tianeptine's acute opioid effects are also not contingent on Barr2 signaling. These results suggest that understanding the difference in abuse potential between

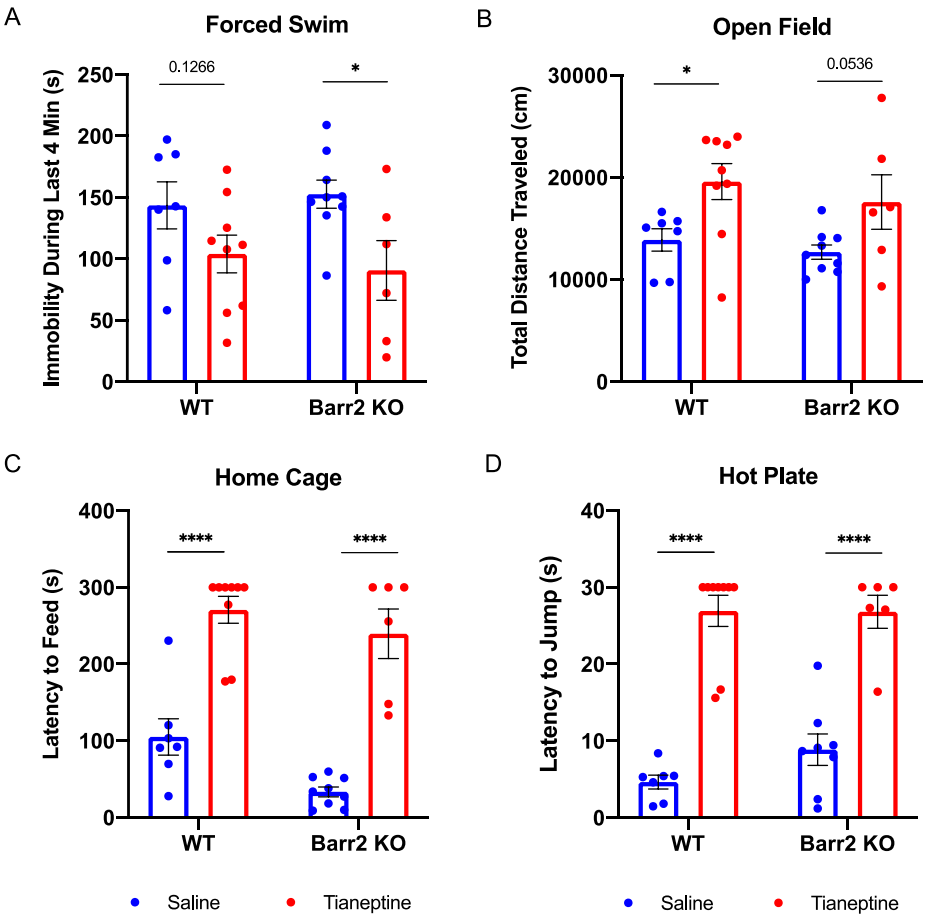

Figure 21: Barr2 expression is not required for tianeptine's acute behavioral effects A) Immobility during the last 4 minutes of the FST. Two-way ANOVA: main effect of treatment: p=0.0064. Planned comparisons, saline vs. tianeptine: p=0.1266 for WT; *p=0.0232 for Barr2 KO (unpaired t-test). B) Open field hyperactivity. Two-way ANOVA: main effect of treatment: p=0.0026. Planned comparisons, saline vs. tianeptine: *p=0.1219 for WT; p=0.0535 for Barr2 KO (unpaired t-test). C) Home cage feeding. Two-way ANOVA: main effect of treatment: p<0.0001, main effect of genotype: p=0.0151. Planned comparisons, saline vs. tianeptine: ****p<0.0001 for WT; ****p<0.00001 for Barr2 KO (unpaired t-test). D) Hot plate analgesia. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: ****p<0.000001 for WT; ****p<0.0001 for Barr2 KO (unpaired t-test). N=6-9, males and females. All bar graphs indicate mean±SEM.

morphine and tianeptine will require parsing downstream signaling pathways with even greater granularity, particularly given mounting evidence that the therapeutic and adverse effects of opioids cannot be neatly decoupled along the lines of G protein vs. ß-arrestin signaling (see Introduction for more detailed discussion).

Notably, prior research has also found that Barr2 KO mice displayed a reduced response to fluoxetine in multiple tasks, suggesting that ß-arrestin 2 signaling is necessary for fluoxetine's antidepressant-like effects[221]. These results are not directly comparable to ours, as they involved chronic, rather than acute, drug administration, and show significant effects in the light-dark and NSF tests, rather than the FST. Nevertheless, they also suggest yet another possible mechanistic distinction between tianeptine and fluoxetine.

3.4 Cell-Type Specificity

3.4.1 MORs on GABAergic Cells are Necessary for the Tianeptine's Acute and Chronic Antidepressant-like effects

We have firmly established that tianeptine requires MORs for its antidepressant effects, but because MOR is widely expressed throughout the brain[84], understanding tianeptine's molecular- and circuit-level mechanisms will require identifying the specific subpopulations of MOR-expressing cells it interacts with. In the hippocampus, MORs are primarily expressed on GABAergic interneurons[230-232], so that was the first population we examined.

By crossing a floxed-MOR line to mice expressing Cre under the VGAT promoter (Figure 22A), we selectively deleted MOR from GABAergic cells and measured the behavioral

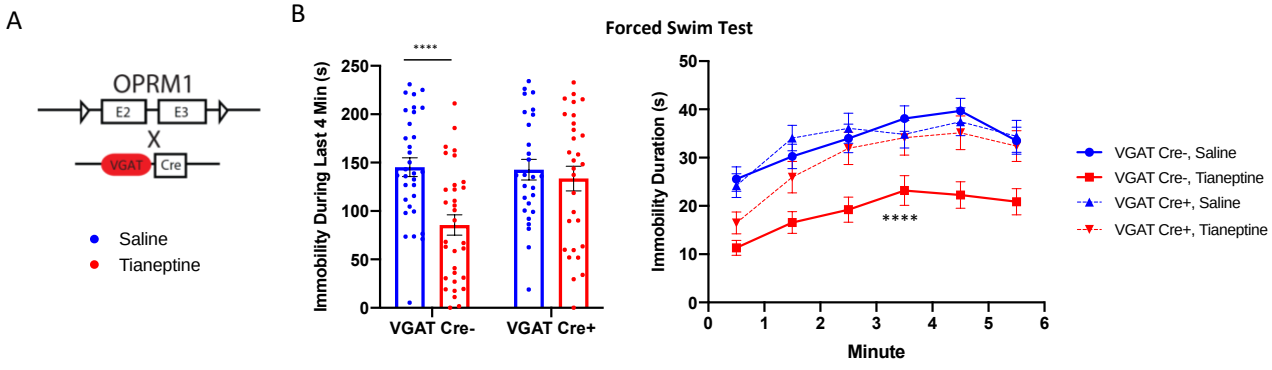

Figure 22: Tianeptine requires MORs on GABAergic neurons for its acute antidepressant-like effects. A) MOR was selectively deleted from GABAergic cells by crossing MOR-floxed mice, which have exon 2/3 of their MOR gene flanked by LoxP sites, to mice expressing Cre recombinase driven by the VGAT promotor. B) FST results. (Left) bar graph shows combined immobility results of last four minutes. Two-way ANOVA: p=0.022 for treatment × genotype. Post hoc t-test, saline vs tianeptine: ****p<0.0001 for VGAT Cre-; p=0.584 for VGAT Cre+. (Right) Line graph shows immobility per minute over the 6-min test. Three-way ANOVA (Time × genotype × treatment): p=0.031 for treatment × genotype. Post hoc repeated measures two-way ANOVA, saline vs tianeptine: ****p<0.0001 for VGAT Cre- and p=0.269 for VGAT Cre+.

response to acute and chronic tianeptine. In the FST, tianeptine decreased immobility time in Cre- but not in Cre+ mice, suggesting that MOR expression on GABAergic neurons is necessary for tianeptine's acute antidepressant action (Figure 22B).

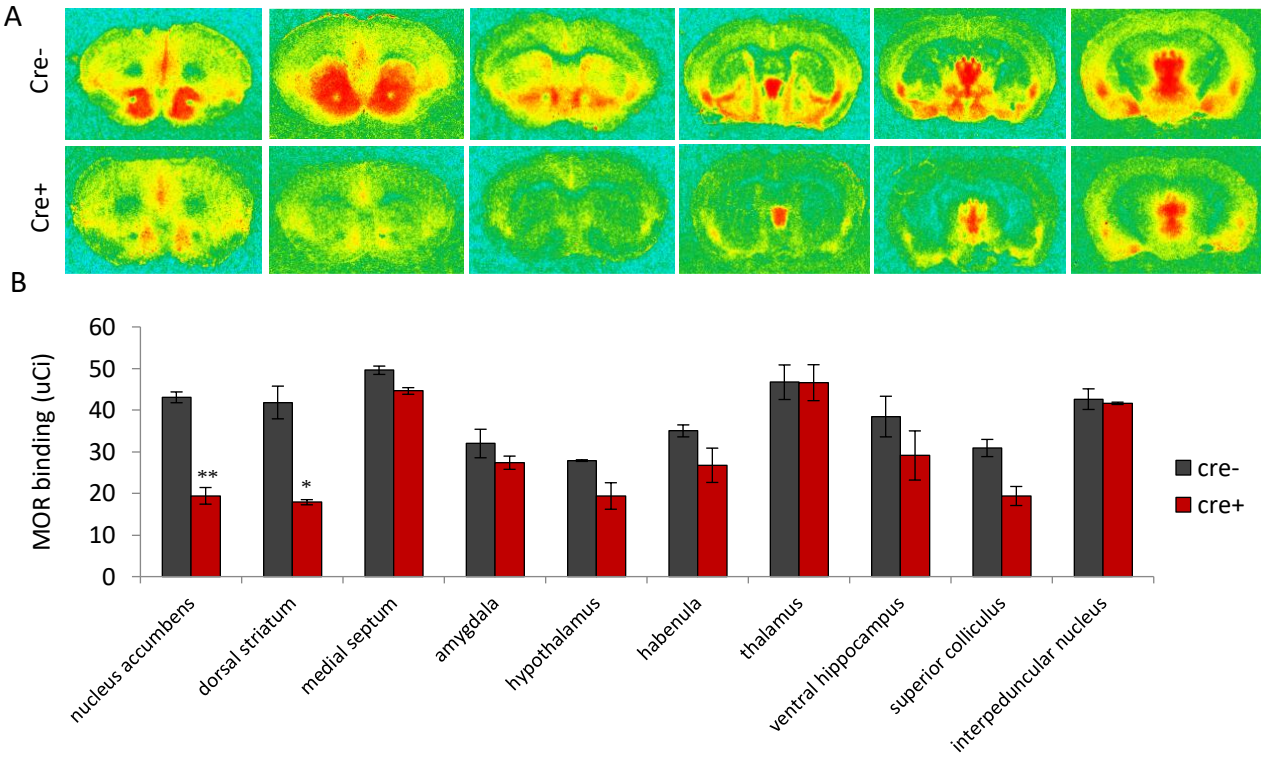

Figure 23: MOR-floxed VGAT Cre+ mice exhibit targeted depletion of MOR compared to Cre- controls. A) ^{3}[H]DAMGO autoradiography for MOR binding was done to confirm conditional knockout of MOR proteins. Shown here are representative brain sections from a Cre+ and Cre- mouse. B) Quantification of MOR binding in various brain regions of interest. Two-way ANOVA: main effect of genotype: p<0.0001; main effect of brain region: p<0.0001; genotype x brain region interaction: p=0.0054. p=0.022 for treatment × genotype. Post hoc t-test, Cre- vs Cre+: **p<0.01 for

nucleus accumbens; *p=0.0265 for dorsal striatum; p=0.0605 for medial septum; p=0.3468 for amygdala; p=0.1165 for hypothalamus; p=0.1995 for habenula; p=0.9879 for thalamus; p=0.3466 for ventral hippocampus; p=0.0647 for superior colliculus; p=0.7266 for interpeduncular nucleus. N=2 mice per genotype.

Selective depletion of MOR was verified by [^{3}H]DAMGO autoradiography on MOR-floxed VGAT Cre+ and Cre- brains, which showed dramatic reductions of MOR binding in regions known to contain MORs on GABAergic cells (Figure 23A). Quantification of MOR binding confirmed that VGAT Cre+ mice showed markedly less MOR binding in multiple brain areas, most notably in the nucleus accumbens and dorsal striatum (Figure 23B). It should be kept in mind, however, that the autoradiography results obtained with this particular ligand may not be of high enough resolution to detect finer reductions in MOR expression that are limited to smaller populations and/or specific cell types. Nevertheless, they do present several candidate regions for tianeptine's site of action (although structures where a significant reduction is not observed may still be involved in tianeptine's antidepressant-like action) and demonstrate the efficacy of our targeted MOR KO.

In addition to tianeptine's antidepressant-like effects, we also looked at the classic opioid-like responses to tianeptine. The rewarding properties of tianeptine were assessed using the conditioned place preference (CPP) paradigm. Here, we found that tianeptine induced a preference in both genotypes, although planned comparisons were only significant in the VGAT Cre- mice (Figure 24A). Similarly, in both genotypes, tianeptine produced acute analgesic effects in the hot plate test, as evidenced by a significant increase in the latency to jump when placed on a heated surface (Figure 24B), and induced hyperlocomotion in the open field test, indicated by an increase in total distance traveled (Figure 24D). Interestingly, tianeptine decreased home cage feeding in Cre- but not in Cre+ mice, suggesting that the opioid-like acute hypophagic response

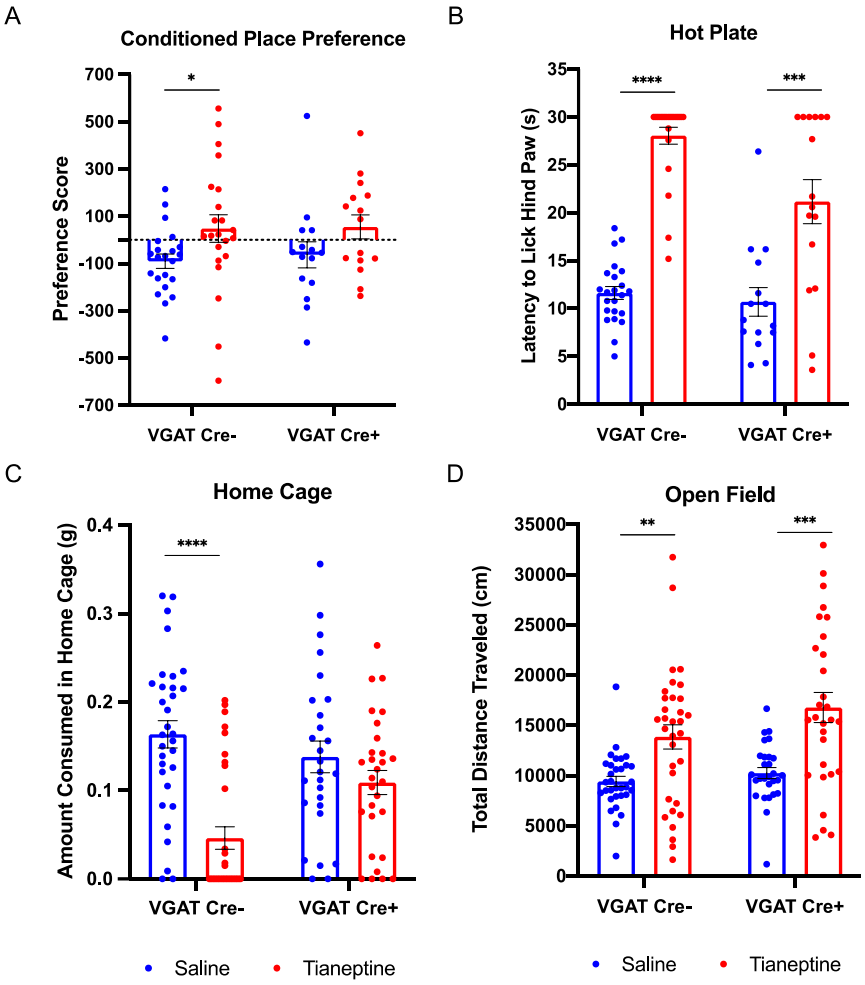

Figure 24: Tianeptine does not require MORs on GABAergic neurons for most of its acute opioid-like effects. A) The conditioned place preference paradigm was used to test the rewarding effects of tianeptine. The preference score (time spent in drug-paired side – time spent in control side) after 8 days of context pairings with tianeptine (30 mg/kg) or saline is shown. Two-way ANOVA: main effect of treatment: p=0.013. Planned comparison, saline vs tianeptine: *p=0.041 for VGAT Cre-; p=0.129 for VGAT Cre+ (unpaired t-test). B) Analgesia was assessed using latency to lick hind paw after being placed on the hot plate (15 min post injection with 30 mg/kg tianeptine, i.p). Two-way ANOVA: main effect of treatment, p<0.0001; p=0.027 for treatment × genotype. Post hoc t-test, saline vs tianeptine: ****p<0.000001 for VGAT Cre-; ***p<0.001 for VGAT Cre+. C) Home cage feeding over 5 min after an 18-h deprivation period was assessed as a measure of hypophagia. Two-way ANOVA: main effect of treatment: ****p<0.0001; p=0.004 for treatment × genotype. Post hoc t-test, saline vs tianeptine: ****p<0.000001 for VGAT cre-; p=0.200 for VGAT cre+. D) Hyperactivity was assessed using total distance traveled in an open field box over 30 min. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: **p=0.001 for VGAT Cre-; ***p<0.001 for VGAT Cre+. N=28-33 per group for (B,D,F) and 15-22 per group for (C,E). Males and females. All acute behavioral assays except hot plate were conducted 1 h after an acute i.p. injection of tianeptine.

to tianeptine requires MORs on GABAergic cells (Figure 24C). Overall these data show that while the acute antidepressant-like and hypophagic effects of tianeptine require GABAergic MOR expression, the other acute opioid-like effects may not—at least not in a manner captured by this cross. Although these results do not allow us to definitively rule out GABAergic neurotransmission as a mechanism underlying MOR-induced hyperactivity, analgesia, and reward (particularly given the extensive literature implicating GABAergic cells in all of these processes), they crucially suggest a dissociation in the mechanisms of action for the acute antidepressant-like and opioid-like effects of tianeptine.

When we divided the animals by sex, we noticed that tianeptine's apparent dependence on MORs for its acute antidepressant-like effects was more noticeable for male than for female

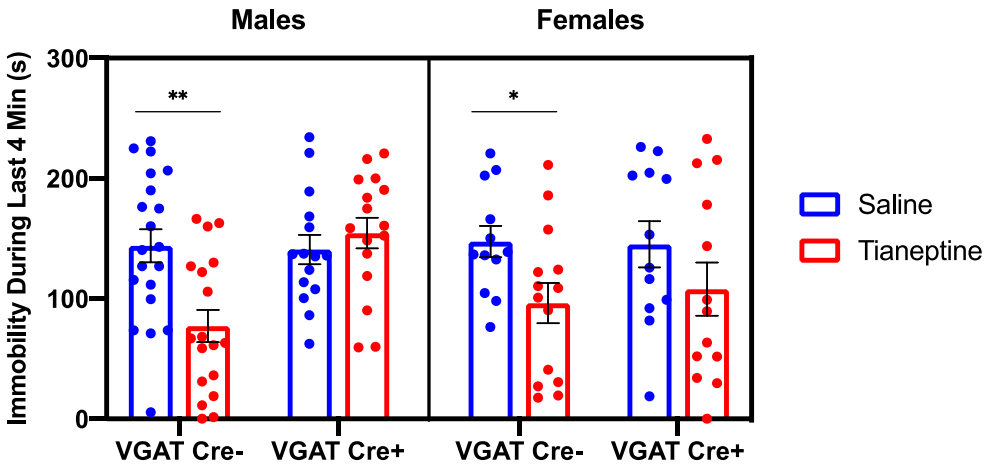

Figure 25: Role of GABAergic MORs in tianeptine's acute antidepressant-like effects for male and female mice. FST results segregated by sex. Bar graphs show combined immobility results of last four minutes. Three-way ANOVA: main effect of genotype: p=0.0017; genotype x drug interaction: p=0.0342. (Left) Males. Two-way ANOVA main effects: p=0.0070 for genotype; p=0.0050 for drug; significant genotype x drug interaction: p=0.0036. Post hoc t-test, saline vs. tianeptine: **p=0.0013 for VGAT Cre+, p=0.4479 for VGAT Cre-. N=15-20 mice per group (Right) Females. Two-way ANOVA: significant main effect of drug: p=0.0190. Planned comparisons, saline vs. tianeptine: *p=0.0268 for VGAT Cre-, p=0.2187 for VGAT Cre-. N=12-14 mice per group.

mice. As seen in the combined sex data, VGAT Cre- male mice treated with tianeptine show clearly reduced immobility in the FST, whereas VGAT Cre+ males do not (Figure 25, left). Statistically, tianeptine only has significant antidepressant-like effects in VGAT Cre- mice in females as well, but the magnitude of this difference is lower, and by eye tianeptine shows a trend towards reducing immobility times in both genotypes for female mice (Figure 25, right). It may be of interest to replicate this experiment in the future with larger cohorts to see if a significant sex difference emerges, as no definite conclusions can be drawn from this preliminary analysis.

We next looked for potential sex differences in the opioid-like responses to tianeptine. In the CPP test, tianeptine tends to slightly increase preference for the drug paired condition across the board, although nothing was statistically significant (Figure 26A). For home cage feeding, both males and females show acute hypophagia, which is lost in the VGAT Cre+ mice (Figure 26B). While tianeptine appears to increase paw-lick latency in the hot plate test regardless of genotype, the magnitude of this analgesic effect is notably lower in VGAT Cre+ males compared to all other groups; indeed, the saline vs. tianeptine difference for these mice does not reach statistical significance (Figure 26C). It should be kept in mind, however, that the CPP and hot plate tests include fewer animals than the other two (they were done on 2 cohorts of mice rather than 3), so the lack of significance in some groups might simply be the result of insufficient statistical power.

In the open field test, tianeptine tends to increase locomotion for all mice, but this effect is surprisingly not significant in the VGAT Cre- mice (Figure 26D). Tianeptine also appears to promote hyperactivity more strongly in females than for males, though whether this is a real difference or simply an artifact is unclear (Figure 26D). There even the suggestion of a

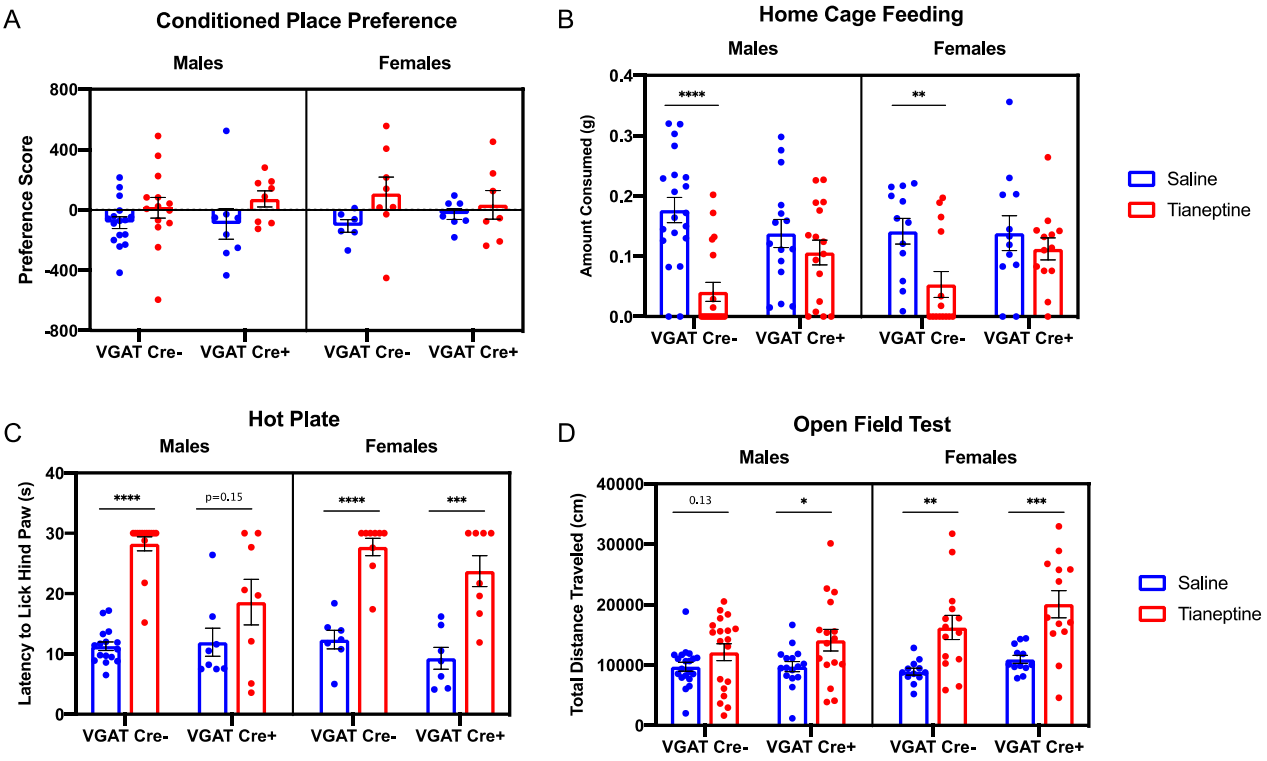

Figure 26: Role of GABAergic MORs in tianeptine's acute opioid-like effects for male and female mice. A) CPP. The preference score is calculated as time spent in drug-paired side minus time spent in control side after 8 days of context pairings with tianeptine (30 mg/kg, i.p.). (Left) Males. No significant effects, although p=0.0561 for the main effect of drug (Two-way ANOVA). (Right) Females. No significant effects. B) Home cage feeding. (Left) Males. Two-way ANOVA: main effect of drug: p=0.0001, drug x genotype interaction: p=0.0122. Post hoc t-tests, saline vs. tianeptine: ****p<0.00001 for VGAT Cre-, p=0.3201 for VGAT Cre+. (Right) Females. Two-way ANOVA: main effect of drug: p=0.0147. Planned comparisons, saline vs. tianeptine: **p=0.0077 for VGAT Cre-, p=0.4447 for VGAT Cre+ (unpaired t-tests). C) Hot plate. (Left) Males. Two-way ANOVA: main effects of genotype (p=0.0181) and drug (p<0.0001), significant genotype x drug interaction: p=0.0074. Post hoc t-tests, saline vs. tianeptine: ****p<0.000001 for VGAT Cre-, p=0.1548 for VGAT Cre-. (Right) Females. Two-way ANOVA: main effect of drug: p<0.0001. Planned comparisons, saline vs. tianeptine: ****p<0.00001 for VGAT Cre-, ***p=0.0006 for VGAT Cre+. D) Open field test. (Left) Males. Two-way ANOVA: main effect of drug: p=0.0081. Planned comparisons, saline vs. tianeptine: p=0.1323 for VGAT Cre-, *p=0.0346 for VGAT Cre+ (unpaired t-tests). (Right) Females. Two-way ANOVA: main effect of drug: p<0.0001. Planned comparisons, saline vs. tianeptine: **p=0.0030 in VGAT Cre- mice, ***p<0.001 for VGAT Cre+ mice. (A,C) N=8-16 per group in males and N=6-8 per group in females (B,D) N=15-20 mice per group, N=12-14 per group. All bar graphs indicate mean±SEM.

somewhat bimodal distribution of travel distances for male Cre- mice (which again, could be attributed to noise in the data), reminiscent of the pattern seen when a population is divided into drug responders and non-responders.

Having established that MORs on GABAergic cells are required for the acute antidepressant-like effect of tianeptine (at least for males), we next investigated whether this receptor population also mediates tianeptine's chronic antidepressant-like effects. Using the chronic corticosterone model (Figure 27A), we observed a trend in which tianeptine lowered

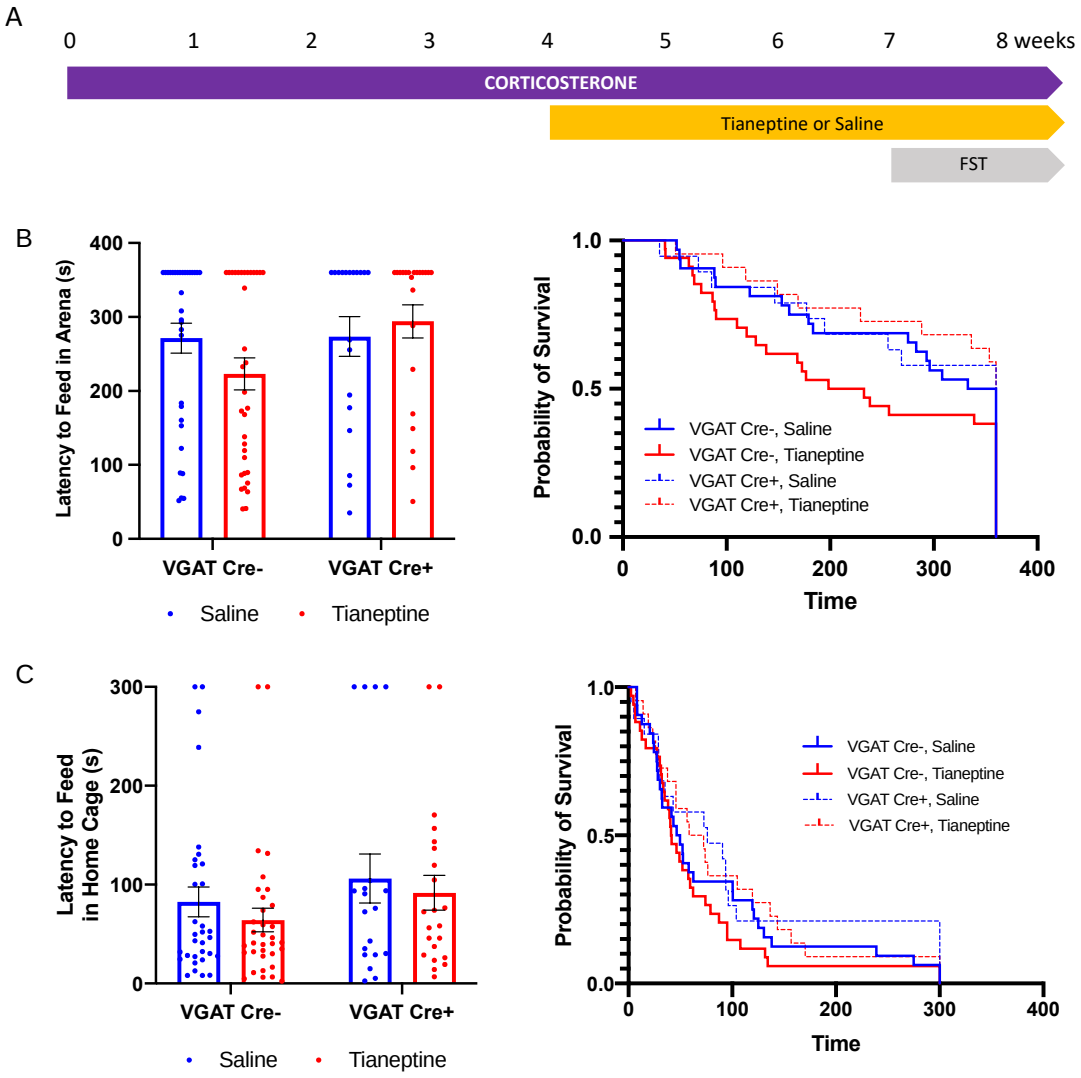

Figure 27: Tianeptine may require MORs on GABAergic cells for its chronic antidepressant-like effects. A) Timeline for chronic treatment. N=19-34 per group. Tianeptine (30 mg/kg, i.p.) was administered twice daily for 3 weeks to chronic corticosterone-treated mice. B) NSF results. Latency to feed in the novel arena (18 h post injection) expressed as a bar graph (left) and survival curves (right). Planned comparisons, saline vs tianeptine: p=0.209 for VGAT Cre-; p=0.827 for VGAT Cre+ (Logrank). (I) Latency to feed in the home cage was measured following the arena test for VGAT Cre- and VGAT Cre+ mice. Logrank (Mantel–Cox Survival): p=0.333. Post-hoc logrank test, saline vs tianeptine: p=0.6936 for VGAT Cre-; p=0.4372 for VGAT Cre+. Each dot represents an individual mouse. All bar graphs indicate mean±SEM.

latency to feed in Cre- but not Cre+ mice, although this difference was not significant (Figure 27B). Once again, tianeptine did not significantly affect feeding latency in the familiar home cage, confirming that the results of the arena test are not confounded by hunger (Figure 27C). Given the previously reported sex differences in the corticosterone stress paradigm[361], we analyzed the data separated by sex and found that in males, tianeptine decreased latency to feed in Cre- but not in Cre+ mice (Figure 28A). In females, however, tianeptine did not have an effect

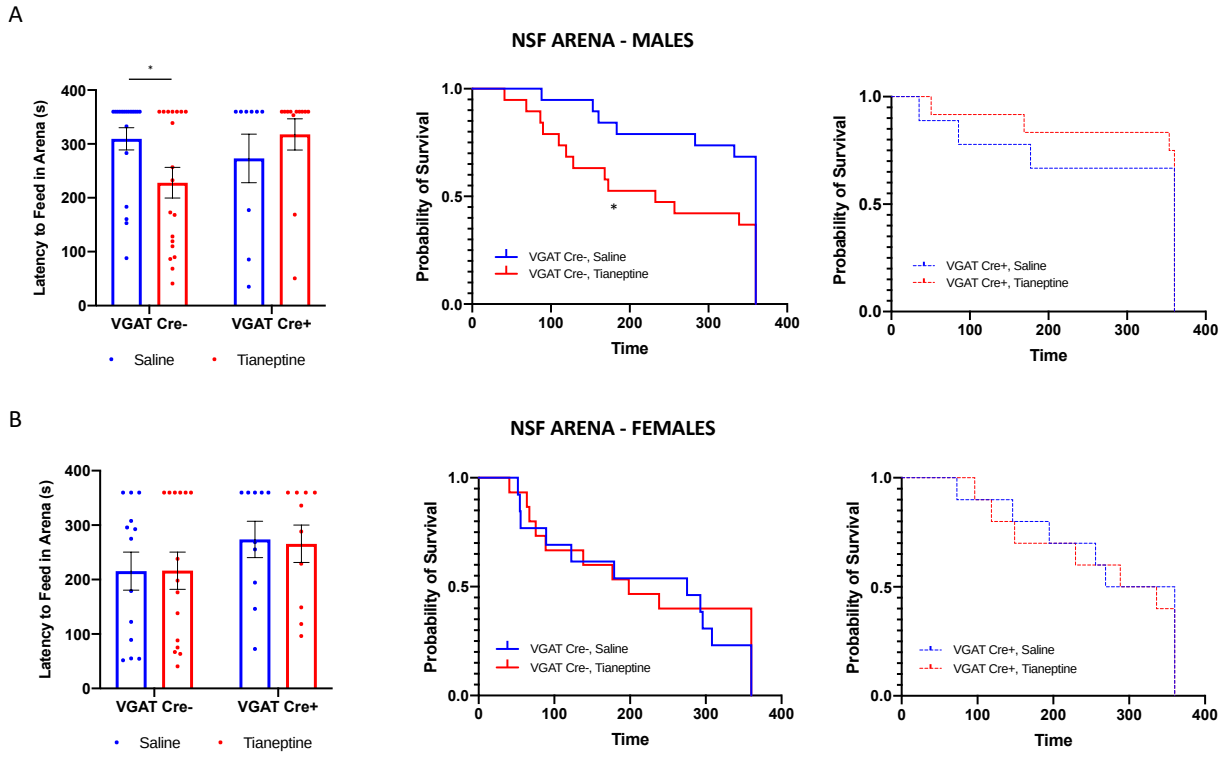

Figure 28: GABAergic MORs are necessary for the chronic antidepressant-like effects of tianeptine in male, but not female, mice. A) Latency to feed in the novel arena following chronic treatment with tianeptine (30 mg/kg twice daily for 3 weeks) in male chronic corticosterone-treated mice. Logrank (Mantel–Cox Survival): p=0.072. Post-hoc logrank test, saline vs tianeptine: *p=0.035 for VGAT Cre-; p=0.632 for VGAT Cre+. B) NSF latency in female mice. Logrank (Mantel–Cox Survival): p=0.616. Post-hoc logrank test, saline vs tianeptine: p=0.605 for VGAT Cre-; p=0.749 for VGAT Cre+.

in either VGAT Cre+ or Cre- mice (Figure 28B). This apparent lack of antidepressant-like efficacy in female mice following chronic tianeptine is likely due to females being less susceptible to the corticosterone paradigm, as described by Mekiri *et al.*[361]. In Figure 28B, it is clear that the baseline NSF latency is much lower for female Cre- mice treated with saline than for male Cre- mice treated with saline, suggesting that tianeptine's chronic effect in female Cre- mice was probably masked by a floor effect. Nevertheless, it is not impossible that the data reflect a true sex difference in response to tianeptine. Overall, at least for male mice, GABAergic MORs may be necessary for both the acute and chronic antidepressant-like effects of tianeptine.

3.4.2 MORs on D1 Cells are not Required for Tianeptine's Acute Antidepressant-like Effects

To identify a specific population of GABAergic cells involved in mediating tianeptine's antidepressant-like effects, we targeted subsets of GABAergic neurons using additional Cre mouse lines. Most GABAergic cells are locally projecting interneurons, but there are a few classes of long-range GABAergic cells, most notably the medium spiny neurons (MSNs) of the striatum[366]. To identify a more specific population of GABAergic cells involved in mediating tianeptine's antidepressant effects, the next step was to target subsets of GABAergic neurons using additional Cre mouse lines.

Given that altered reward processing is a hallmark of depression[367], and the pronounced loss of MORs in the striatum of MOR-floxed VGAT Cre mice (Figure 23), it was

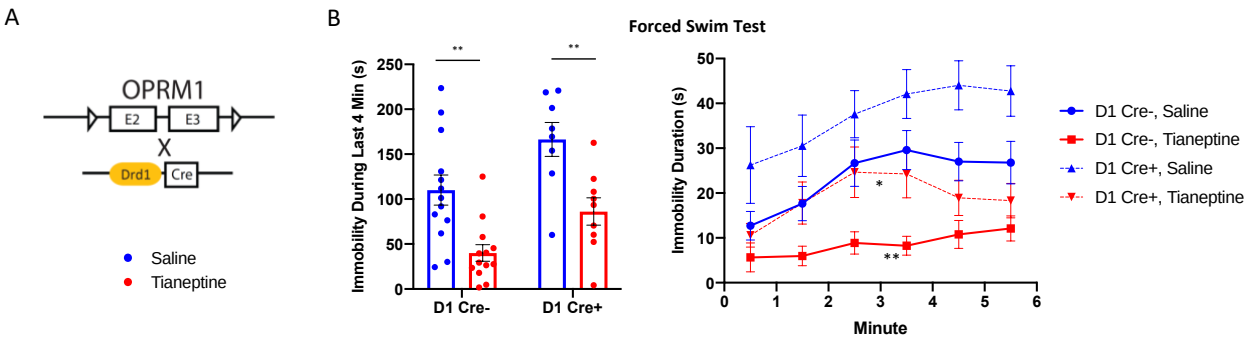

Figure 29: Tianeptine does not require MORs on D1 cells for its acute antidepressant-like effects. A) MOR was selectively deleted from D1 cells by crossing MOR-floxed mice to mice expressing Cre recombinase driven by the D1 promotor. B) FST results. (Left) bar graph shows combined immobility results of last four minutes. Two-way ANOVA: main effect of treatment: $p<0.0001$. Main effect of genotype: $p=0.0018$ Planned comparisons, saline vs tianeptine: **p=0.001 for D1 Cre-; **p= 0.004 for D1 Cre+ (unpaired t-test). (Right) Line graph shows immobility per minute over the 6-minute test. Planned comparisons, saline vs tianeptine: **p=0.002 for D1 Cre- and *p=0.011 for D1 Cre+ (repeated measures two-way ANOVA). N=8-13 mice per group.

possible that D1 or D2 MSNs might mediate tianeptine's antidepressant-like effects. D1 seemed like an especially promising target, in light of 2014 paper which tested whether targeted re-expression of MOR in D1 MSNs could restore key opiate-driven behaviors that are absent in MOR knockout mice, such as the rewarding properties of morphine in the conditioned place preference (CPP) paradigm. Using two independent cohorts of mice, the authors showed a lack of CPP in MOR KO mice and restoration of CPP to WT control levels in "rescue" mice that selectively re-expressed MOR only in DI MSNs, demonstrating that selective re-expression of MOR in D1 cells is sufficient to restore morphine-induced reward *in vivo*.

When we targeted these neurons using MOR-floxed D1-Cre mice (Figure 29A), we found that in mice lacking MOR on D1+ cells, tianeptine still produced a robust antidepressant-like effect, as evidenced by decreased immobility in the FST (Figure 29B). The majority of tianeptine's opioid-like effects were also still intact, including conditioned place preference

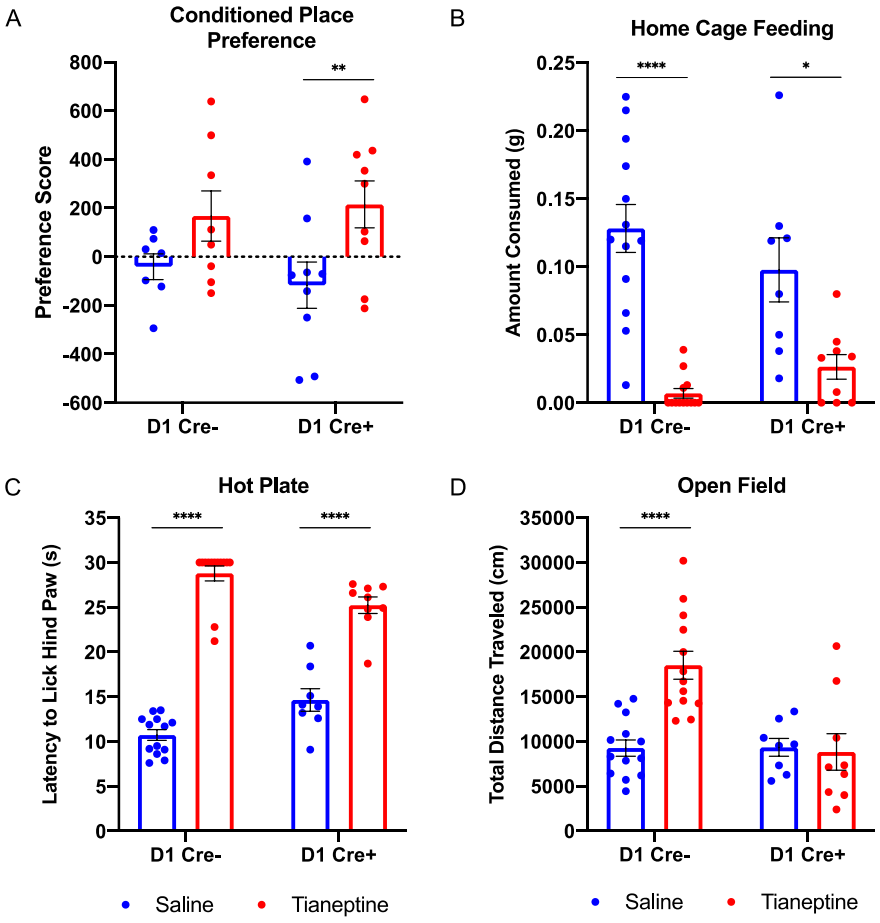

Figure 30: Tianeptine does not require MORs on D1 cells for most of its acute opioid-like effects. A) The conditioned place preference paradigm was used to test the rewarding effects of tianeptine. The preference score (time spent in drug-paired side – time spent in control side) after 8 days of context pairings with tianeptine (30 mg/kg) or saline is shown. Two-way ANOVA: main effect of treatment: p=0.007. Planned comparison, saline vs tianeptine: p=0.109 for D1 Cre-; p=0.026 for D1 Cre+ (unpaired t-test). B) Home cage feeding over 5 min after an 18-h deprivation period was assessed as a measure of hypophagia. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: ****p<0.000001 for D1 Cre-; *p=0.010 for D1 Cre+ (unpaired t-test). C) Analgesia was assessed using latency to jump after being placed on the hot plate (15 min post injection). Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: ****p<0.000001 for D1 Cre-; ****p<0.00001 for D1 Cre+ (unpaired t-test). D) Open field hyperlocomotion results. Two-way ANOVA: p=0.002 for treatment × genotype. Post-hoc t-tests, saline vs tianeptine: ****p<0.0001 for D1 Cre-; p=0.829 for D1 Cre+. N=8-13 mice per group. All acute behavioral assays except hot plate were conducted 1 h after an acute i.p. injection of tianeptine.

reward (Figure 30A), acute hypophagia (Figure 30B), and analgesia (Figure 30C). Tianeptine did not, however, induce hyperlocomotion in the open field for D1 Cre+ mice (Figure 30D).

The persistence of CPP in our D1 Cre+ mice seems to contradict the earlier results from Cui *et al*. showing the necessity of MOR expression on D1 MSNs for the development of morphine CPP. There are multiple explanations that could account for this incongruity, which are discussed at length in the Discussion. Most notably, our experiments tested the *necessity* of MORs on D1 cells whereas Cui *et al*. addressed the question of *sufficiency*. Thus, it is possible to imagine a scenario where MORs on multiple cell types are independently capable of mediating reward, such that targeted re-expression of MORs on D1 MSNs produces CPP, while deletion of D1 MORs does not abolish it (due to compensation from other cell populations).

3.4.3 MORs on SST, but not PV, Cells are Required for the Acute and Chronic Antidepressant-like Effects of Tianeptine

Next, we considered MORs on somatostatin (SST) and parvalbumin (PV) cells, both of which are major, non-overlapping classes of GABAergic interneurons that express MOR [198,199,232]. To specifically knock down MOR on SST cells, we crossed the MOR-floxed line to mice expressing Cre recombinase driven by the SST promoter (Figure 31A). In the FST,

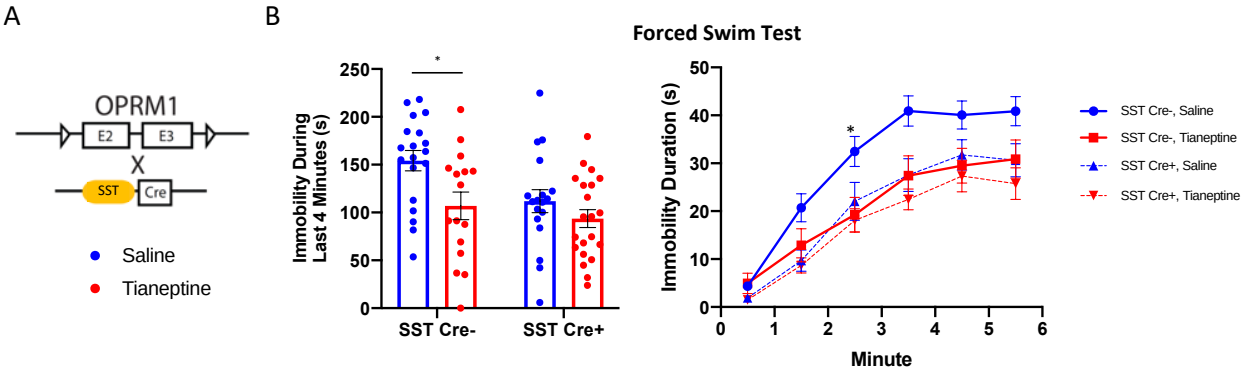

Figure 31: Tianeptine may require MORs on SST neurons for its acute antidepressant-like effects. A) MOR was selectively deleted from SST cells by crossing MOR-floxed mice to mice expressing Cre recombinase driven by the SST promotor. B) FST day 1 results. (Left) bar graph shows combined immobility results of last four minutes. Planned comparisons, saline vs tianeptine: *p=0.011 for SST Cre-; p= 0.231 for SST Cre+ (unpaired t-test). (Right) Line graph shows immobility per minute over the 6-min test. Planned comparisons, saline vs tianeptine: *p=0.021 for SST Cre- and p=0.256 for SST Cre+ (repeated measures two-way ANOVA).

tianeptine significantly reduced immobility time for the SST Cre- but not the SST Cre+ mice, suggesting that MOR expression on SST cells play a role in mediating tianeptine's acute antidepressant-like action (Figure 31B). A baseline genotype difference was also observed between the Cre- and Cre+ groups for this test. Classic opioid-like effects including analgesia

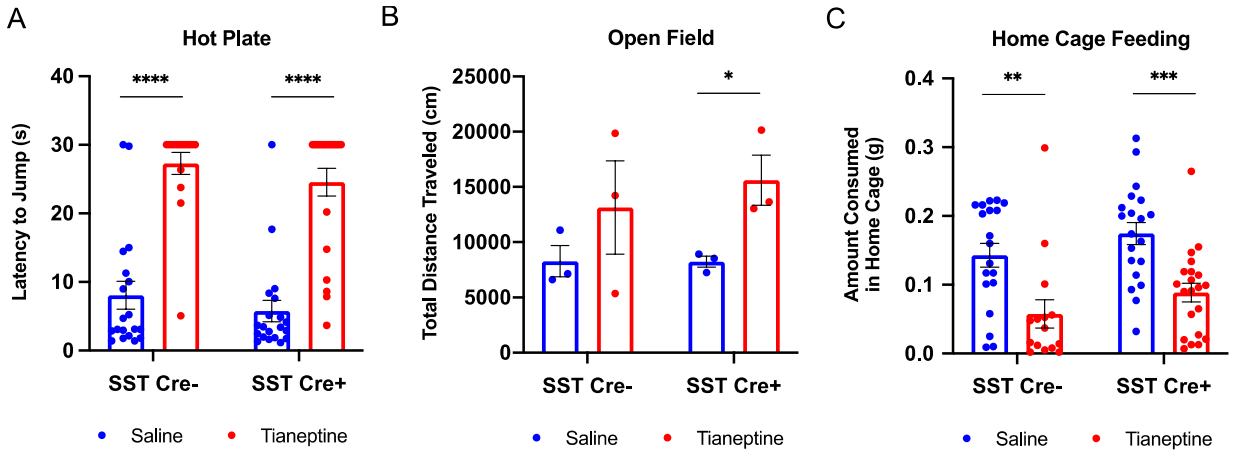

Figure 32: Tianeptine does not require MORs on SST neurons for its acute opioid-like effects. A) Analgesia was assessed using latency to jump after being placed on the hot plate (15 min post injection). Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs. tianeptine: ****p<0.000001 for SST Cre-; ****p<0.000001 for SST Cre+ (unpaired t-test). N=16-21 per group. B) Open field hyperlocomotion results. Two-way ANOVA: main effect of treatment: p=0.041. Planned comparisons, saline vs tianeptine: p=0.336 for SST Cre-; *p=0.034 for SST Cre+. N=3 per group. C) Home cage feeding results. Two-way ANOVA: main effect of treatment: p<0.0001. Planned comparisons, saline vs tianeptine: *p=0.0031 for SST Cre-; p=0.0002 for SST Cre+ (unpaired t-test). N=15-21 per group. All acute behavioral assays except hot plate were conducted 1 h after an acute i.p. injection of tianeptine.

(Figure 32A), hyperactivity (Figure 32B), and hypophagia (Figure 32C), were intact, suggesting that antidepressant-like effects of tianeptine can be dissociated from the classic opioid effects, and likely have different mechanisms of action.

In chronic corticosterone-treated mice (Figure 33A), chronic tianeptine significantly reduced forced swim immobility in SST Cre- but not SST Cre+ mice, suggesting that MOR expression on SST cells is also required for the chronic antidepressant-like effects of tianeptine (Figure 33B). Here, the FST was used in place of NSF as a chronic test because tianeptine did not produce an antidepressant-like effect even in control mice for the NSF test. This is likely due

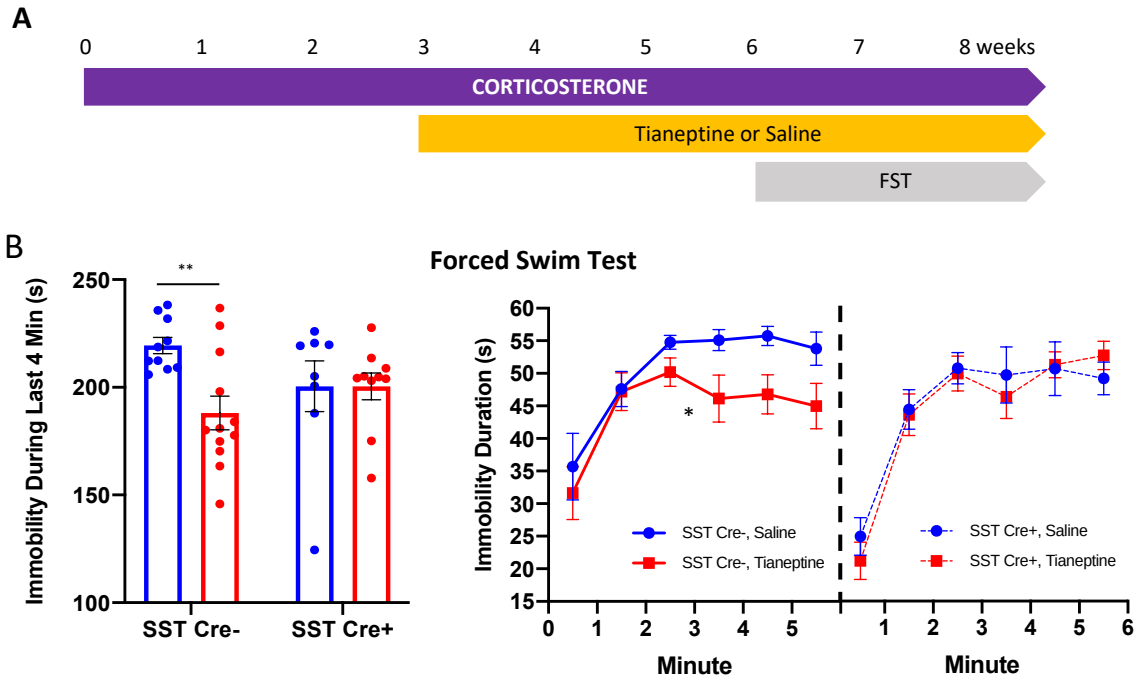

Figure 33: Tianeptine requires MORs on SST neurons for its chronic antidepressant-like effects. A) Timeline for (B). *n*=8-12 per group. Tianeptine (30 mg/kg, i.p.) was administered twice daily for 3 weeks to chronic corticosterone-treated mice. B) FST results. (Left) bar graph shows combined immobility results of last four minutes. Two-way ANOVA: p=0.048 for treatment × genotype. Planned comparisons, saline vs tianeptine: *p=0.011 for SST Cre-; p= 0.231 for SST Cre+ (unpaired t-test). (Right) Line graph shows immobility per minute over the 6-min test. Planned comparisons, saline vs tianeptine: *p=0.021 for SST Cre- and p=0.811 for SST Cre+ (repeated measures two-way ANOVA). Each dot represents an individual mouse. All bar graphs indicate mean±SEM.

to differences in genetic background between the different mixed strains we have been using. Different tests and test conditions are effective in certain strains of mice and not others, and forced swim turned out to be more sensitive to the chronic effects of tianeptine in the genetic background of our SST Cre mice.

We also assessed the necessity of MOR expression on another prominent subset of interneurons which express parvalbumin (PV), again using the Cre-Lox system (Figure 34A). Unlike SST+ cells, MOR expression on PV cells was not necessary to mediate the acute antidepressant-like nor the opioid-like effects of tianeptine (Figure 34B-E).

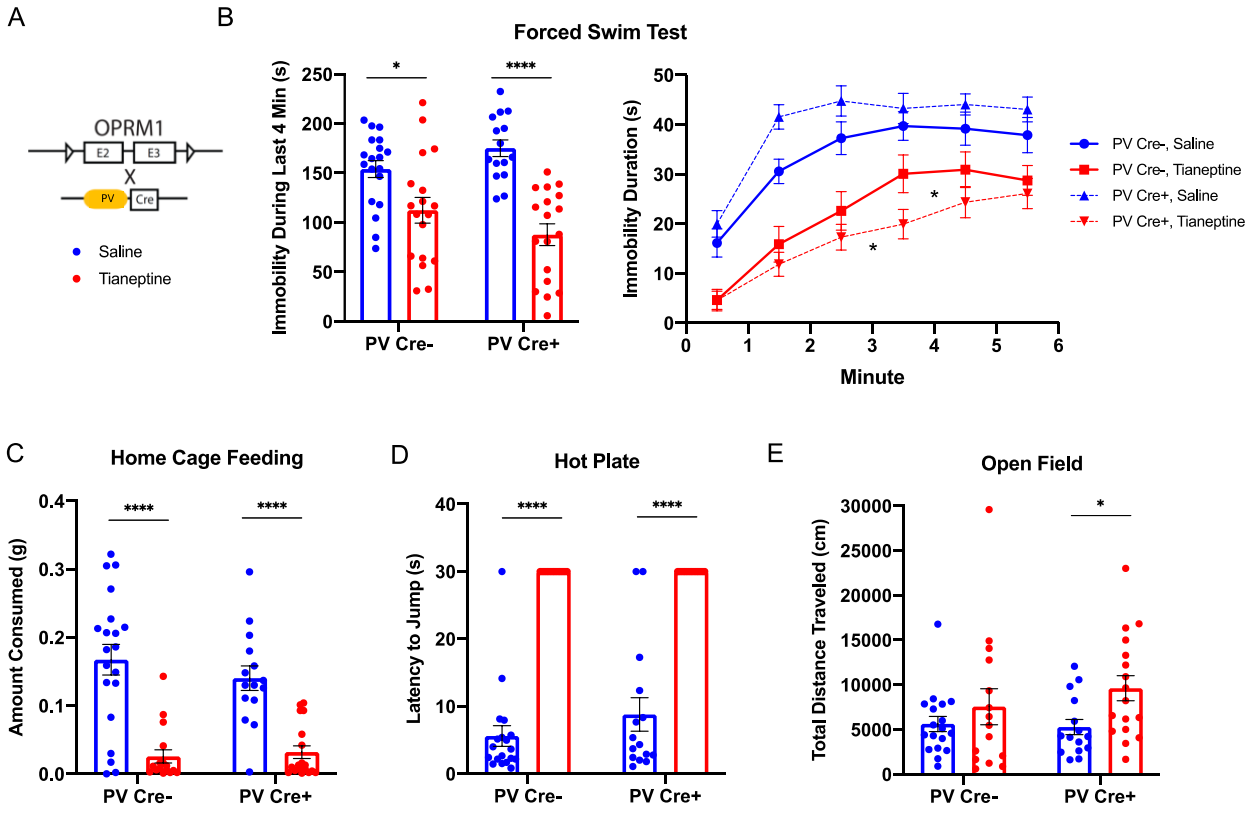

Figure 34: Tianeptine does not require MORs on PV cells for its acute behavioral effects. A) MOR was selectively deleted from PV cells by crossing MOR-floxed mice to mice expressing Cre recombinase driven by the PV promotor. B) FST day 2 results. (Left) bar graph shows combined immobility results of last four minutes. Two-way ANOVA: main effect of treatment: $p<0.0001$. Planned comparisons, saline vs tianeptine: *$p=0.011$ for PV Cre-; ****$p=0<0.000001$ for PV Cre+. (Right) Line graph shows immobility per minute over the 6-minute test. Planned comparisons,

saline vs tianeptine: **p=0.003 for PV Cre- and ****p=0<0.0001 for PV Cre+ (repeated measures two-way ANOVA). C) Home cage feeding over 5 min after an 18-h deprivation period was assessed as a measure of hypophagia. Two-way ANOVA: main effect of treatment: ****p<0.0001; Planned comparisons, saline vs tianeptine: ****p<0.00001 for PV Cre- and ****p<0.00001 for PV Cre+ (unpaired t-test). D) Analgesia was assessed using latency to lick hind paw after being placed on the hot plate (15 min post injection with 30 mg/kg tianeptine, i.p). Two-way ANOVA: main effect of treatment, p<0.0001. Planned comparisons, saline vs tianeptine: ****p<0.000001 for PV Cre- and ****p<0.000001 for PV Cre+ (unpaired t-test). E) Hyperactivity was assessed using total distance traveled in an open field box over 30 min. Two-way ANOVA: main effect of treatment: p<0.022. Planned comparisons, saline vs tianeptine: p=0.355 for PV Cre-; *p<0.016 for PV Cre+. 15-20 mice per group. All acute behavioral assays except hot plate were conducted 1 h after an acute i.p. injection of tianeptine.

3.5 Brain Region Specificity

3.5.1 MORs in the MHb are not Required for Tianeptine's Acute Antidepressant-like Effects

In addition to cell-type specificity, we also investigated which brain regions might be engaged by tianeptine. One promising candidate was the medial habenula (MHb), a structure with a high density of MOR expression[281] and a known role in aversion processing[283]. Interestingly, chronic mild stress in rats was found to significantly alter SST receptor expression and release in the MHb[368], and SST_2 receptor expression in the MHb was later identified as particularly sensitive biomarker for stress-responsiveness[369]. Given our results that MORs on SST cells may be necessary for tianeptine's acute and chronic antidepressant-like effects, the habenula emerges as a strong contender for tianeptine's site of action.

In order to achieve habenula-specific knockdown of MOR, we used the B4 subunit of the nicotinic acetylcholine receptor (Chrnb4, henceforth abbreviated as B4, which is mainly localized to the medial habenula[370]). We crossed B4 Cre+ mice with MOR-floxed mice (Figure 35A) and used confocal imaging of RNAscope® probes targeting mRNAs for *Oprm1* (the gene for MOR) and *Chrnb4* to confirm that the resulting MOR-floxed B4 Cre+ mice

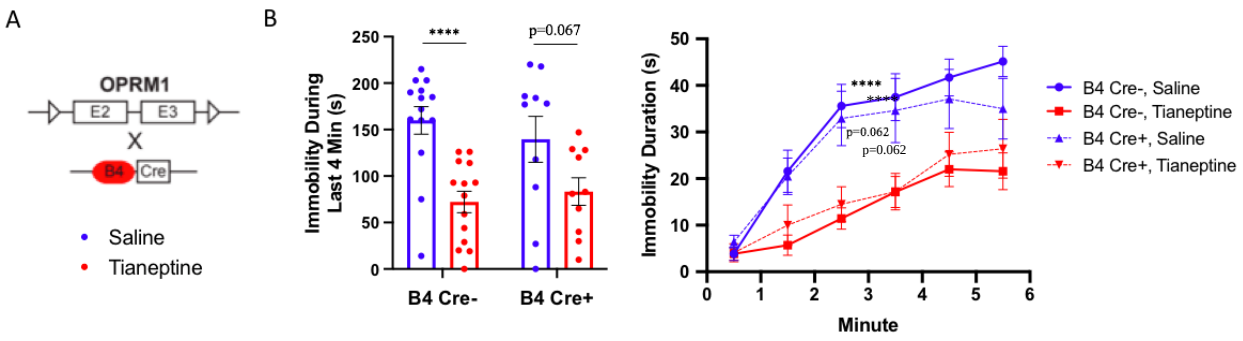

Figure 35: MORs in the habenula are likely not responsible for tianeptine's antidepressant-like effects. A) MORs in the habenula were deleted by crossing MOR-floxed mice, which have exon 2/3 of their MOR gene flanked by *LoxP* sites, to mice expressing Cre recombinase driven by the promotor for the B4 nAChr subunit, which is mainly localized to the medial habenula. B) FST results. (Left) bar graph shows combined immobility results of last four minutes. Two-way ANOVA: main effect of treatment: ****p<0.0001; main effect of genotype: p=0.78; p=0.34 for treatment × genotype. Planned comparisons, saline vs. tianeptine: ****p=0.000079 for Cre-, p=0.066600 for Cre+. (Right) Line graph shows immobility per minute over the 6-min test. Three-way ANOVA (Time as a within subject variable): main effect of treatment: ****p<0.0001; main effect of genotype: p=0.94; treatment x genotype interaction: p=0.38. Planned comparisons, saline vs. tianeptine: ****p<0.0001 for Cre-, p=0.0620 for Cre+ (repeated measures two-way ANOVA).

exhibited habenula-specific reduction of MOR expression, restricted to B4-neurons (Figure 36A)[283]. Surprisingly, in the FST, tianeptine significantly reduced immobility time for both the B4 Cre- and B4 Cre+ mice, suggesting that MORs in the habenula are probably not responsible for the acute antidepressant-like effects of tianeptine (Figure 35B).

However, while habenular expression of MOR is markedly diminished in B4 Cre+ mice compared to controls, some expression still remains (Figure 36A, right); consequently, we cannot entirely exclude the possibility that these residual MOR-positive cells are sufficient to mediate the acute antidepressant-like response to tianeptine. Additionally, while RNAscope possesses several advantages over autoradiography (Figure 23), such as being faster/easier to conduct and offering cellular resolution, one should keep in mind that it reflects *Oprm1* mRNA expression rather than ligand binding to the actual MOR receptor.

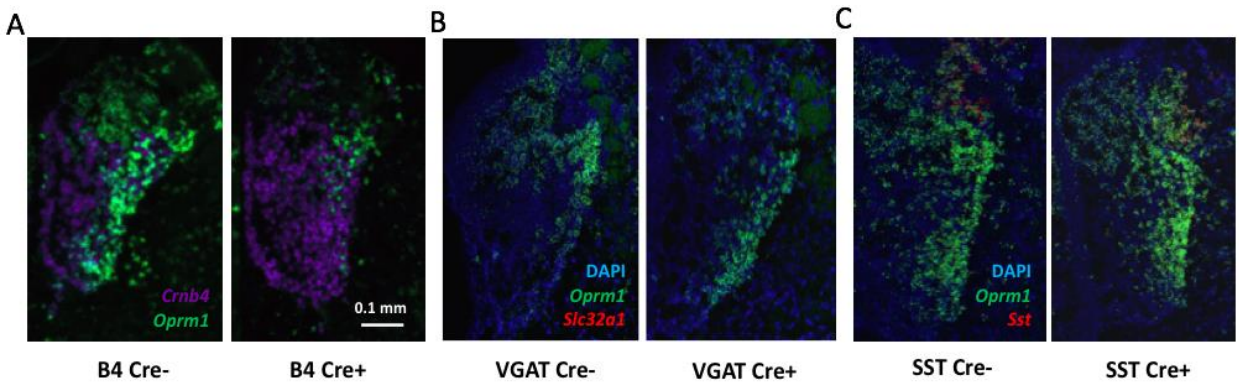

Figure 36: Cellular characterization of MOR expression in the habenula of MOR transgenic mice using RNAscope (ACDbio®). A) Representative confocal images of *Oprm1* (green) and *Crnb4* (purple) transcripts in the medial habenula show markedly decreased expression of *Oprm1* in B4 cre+ mice (right) compared to B4 Cre- mice (left). Data was previously published in [283] B) Representative RNAscope images of *Oprm1* (green) and *Slc32a1* (red) transcripts in the habenula of VGAT Cre- (left) and VGAT Cre+ mice (right) show that expression of *Oprm1* is comparable in both mutant and control mice. *Slc32a1* staining is completely absent in the habenula, regardless of genotype. DAPI stain is shown in blue. C) Representative RNAscope images of *Oprm1* (green) and *Sst* (red) transcripts in the habenula of SST Cre- (left) and SST Cre+ mice (right) show comparable levels of *Oprm1* and *Sst* transcripts in both genotypes. *Oprm1* and *Sst* expression appears to be non-overlapping. DAPI stain is shown in blue.

That said, many of our RNAscope results are largely congruous with both the existing literature and our own behavioral data. The MHb is known to contain mainly glutamatergic neurons[269]; accordingly, RNAscope *in situ* hybridization (ISH) showed a complete absence of *Slc32a1* mRNA (encoding VGAT) within the MHb, and habenular *Oprm1* expression remained unchanged in VGAT Cre+ mice compared to Cre- controls (Fig. 36B). Additionally, *Oprm1* and *Sst* mRNAs appear to be expressed in largely non-overlapping cell populations within the MHb, and MOR expression was comparable in both SST Cre- and Cre+ mice (Fig. 36C). The lack of MOR deletion in the MHb in the two lines of Cre mice in which we observed abolition of tianeptine's antidepressant effects is consistent with the notion that the MHb is not tianeptine's acute site of action.

3.5.2 MORs in the Ventral Hippocampus may be Involved in Tianeptine's Antidepressant-like Effects

Because tianeptine is no longer effective in MOR-floxed VGAT Cre+ and SST Cre+ mice, the brain regions involved should exhibit a marked reduction of MOR expression in both MOR-floxed Cre crosses. The primary structure we examined for this signature is the hippocampus, as it has been extensively implicated in depression[130,214-216] and has significant expression of MORs on GABAergic cells[232].

The hippocampus is thought to be functionally heterogeneous along its longitudinal axis, with the dorsal hippocampus involved in learning and spatial memory, and the ventral hippocampus associated with regulating emotional and motivated behaviors[371]. This is

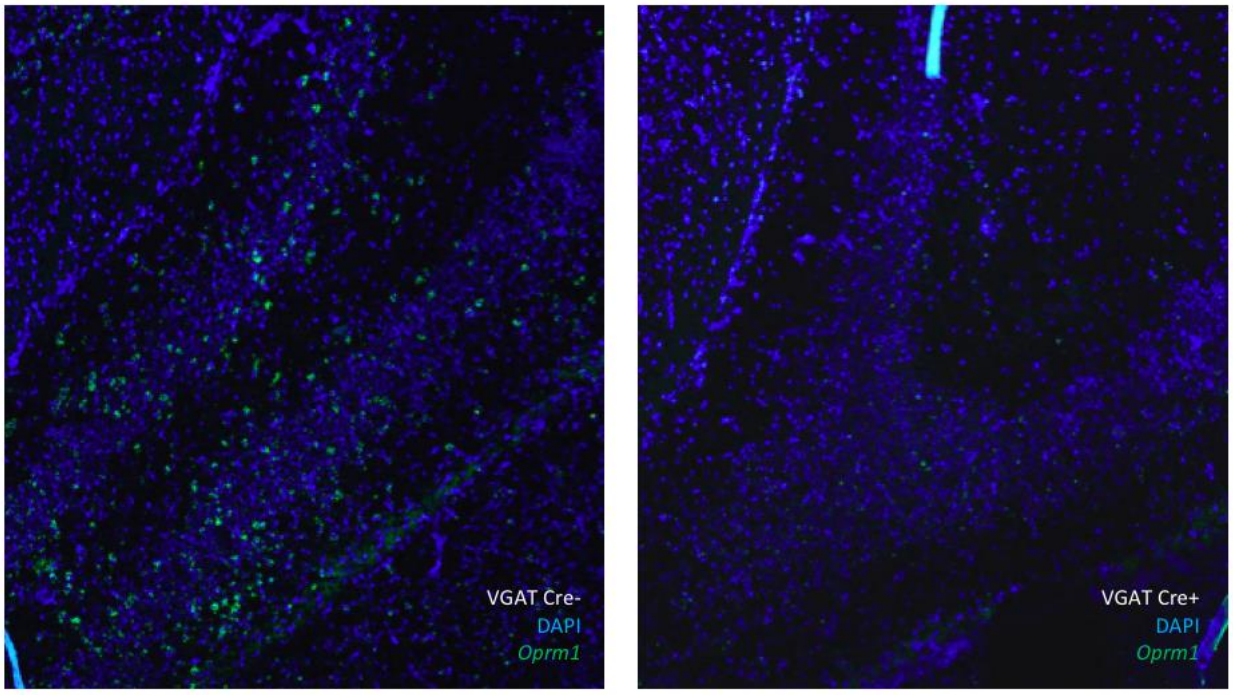

Figure 37: MOR (*Oprm1*) mRNA expression in the ventral hippocampus of MOR-floxed VGAT Cre+ and Cre- mice. Representative confocal images of *Oprm1* (green) transcripts in the ventral hippocampus show markedly decreased expression of *Oprm1* transcripts in VGAT Cre+ mice (right) compared to VGAT Cre- controls (left). DAPI stain is shown in blue.

supported by three major lines of evidence: 1) distinct functional connectivity for the two poles of the hippocampus[372], 2) the dependence of spatial memory on the dorsal, but not the ventral hippocampus[373], and 3) the converse finding that lesions to the ventral but not dorsal hippocampus alter stress responses and emotional behavior[374]. As such, we considered the ventral hippocampus in particular as a potential site of action for tianeptine.

Using RNAscope ISH, we found that *Oprm1* mRNA is abundant in the ventral hippocampus of VGAT Cre- mice, but almost entirely absent in Cre+ mice, suggesting that the vast majority of hippocampal MOR expression occurs on GABAergic cells (Figure 37), as has been reported previously in the literature [232,236,375]. Through double-label RNAscope, we subsequently confirmed that *Oprm1* and *Slc32a1* mRNAs are highly colocalized in ventral hippocampus sections of VGAT Cre- mice, and that *Oprm1* expression on VGAT cells is strongly reduced in VGAT Cre+ mice compared to controls (Figure 38A). Similarly, we observed reduced *Oprm1* transcript expression in SST cells in SST Cre+ mice compared to SST Cre- mice (Figure 38B). Quantification of ISH signals revealed significant reduction of both double *Oprm1*/*Slc32a*-positive neurons in the ventral hippocampus of VGAT Cre+ mice (Figure 38C), and double *Oprm1*/*Sst*-positive cells in the ventral hippocampus of SST Cre+ mice (Figure 38D), indicating a selective loss of *Oprm1* mRNA in GABAergic and SST-expressing neurons, respectively, within the ventral hippocampus. These results point to the possibility that the ventral hippocampus may be involved in mediating the acute and chronic antidepressant-like effects of tianeptine.

While interesting, these results do not definitively implicate the ventral hippocampus as a site of action for tianeptine, as they do not involve any experimental manipulations. To remedy this, we conducted a pilot study assessing the sufficiency of hippocampal MORs for tianeptine's

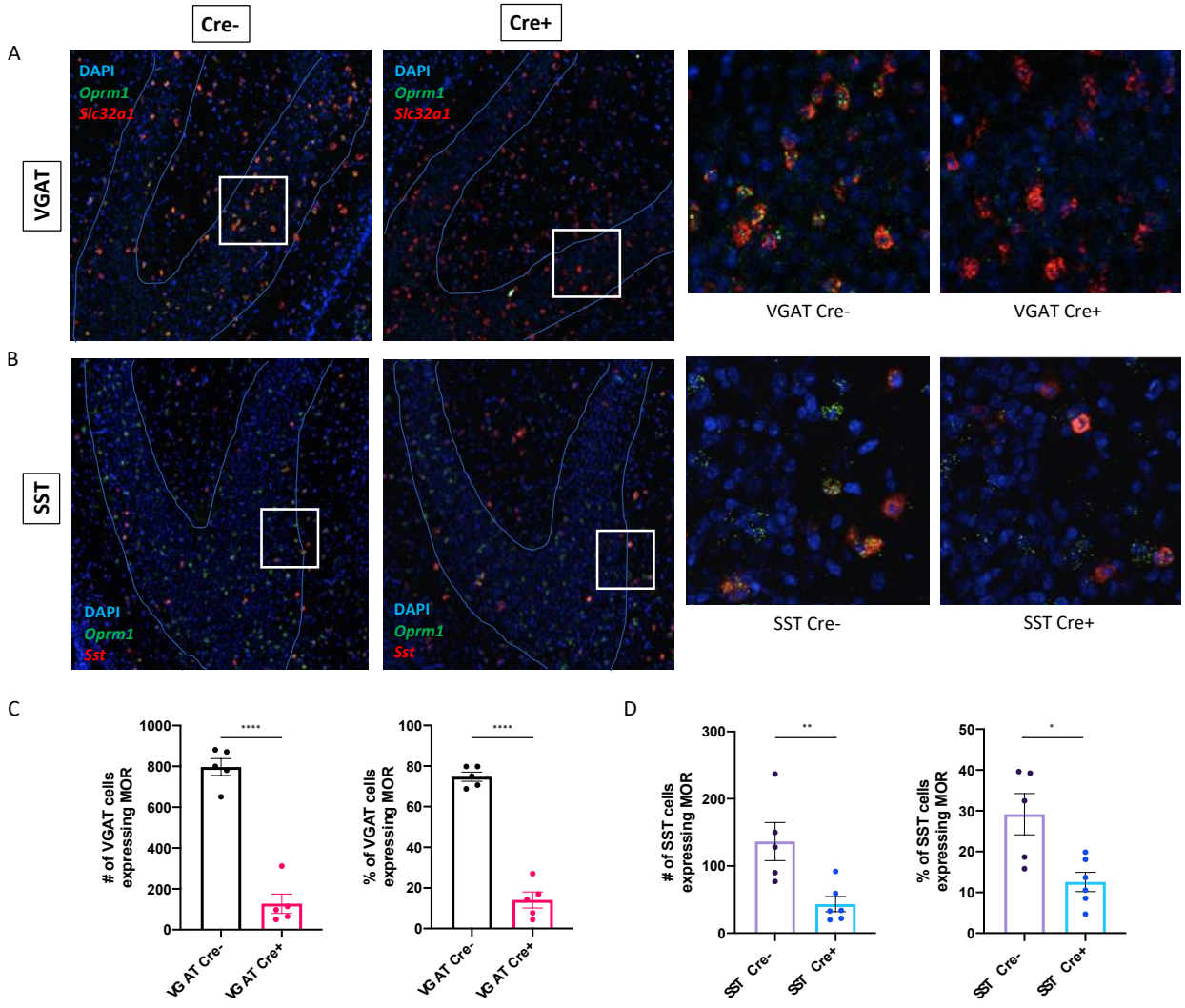

Figure 38: MOR (*Oprm1*) expression is reduced in the ventral hippocampal cells of VGAT and SST Cre+ mice. A) Confocal imaging of RNAscope probes targeting *Oprm1* (green) and *Slc32a1* (red) mRNAs in addition to DAPI staining (blue) shows colocalization of the two transcripts in ventral hippocampus sections. Oprm1 expression on VGAT cells is strongly reduced in VGAT Cre+ mice compared to VGAT Cre- mice. Insets show a magnified image of the area within the white squares B) Representative confocal images of Oprm1 (green) and *Sst* (red) mRNA transcript colocalization shows reduced Oprm1 expression in SST cells in SST Cre+ mice compared to SST Cre- controls. C) Quantification of double *Oprm1*/*Slc32a*-positive cells in the ventral hippocampus of VGAT Cre+ (pink) mice and VGAT Cre- (black) controls. Both the number of *Slc32a*-positive cells that express Oprm1 (left) and the percent of *Slc32a*-positive cells that express *Oprm1* (right) within a section of hippocampus are dramatically lower in VGAT Cre+ mice compared to VGAT Cre- mice (unpaired t-test, ****p<0.0001 for both). D) Quantification of double *Oprm1*/*Sst*-positive cells in the ventral hippocampus of SST Cre+ (cyan) and SST Cre-(purple) mice. Both the number of *Sst*-positive cells that express Oprm1 (left) and the percent of *Sst*-positive cells that express Oprm1 (right) are significantly lower in SST Cre+ mice compared to SST Cre- controls (unpaired t-test, **p<0.01 and *p=0.01, respectively).

acute antidepressant-like effects using central infusions of tianeptine. WT C57BL/6 mice had bilateral cannulae surgically implanted into the ventral hippocampus (Bregma-3.6, ML±2.8, DV-3.5), through which tianeptine was administered 15 minutes prior to behavioral testing in the forced swim and open field assays. We found that central infusion of tianeptine significantly

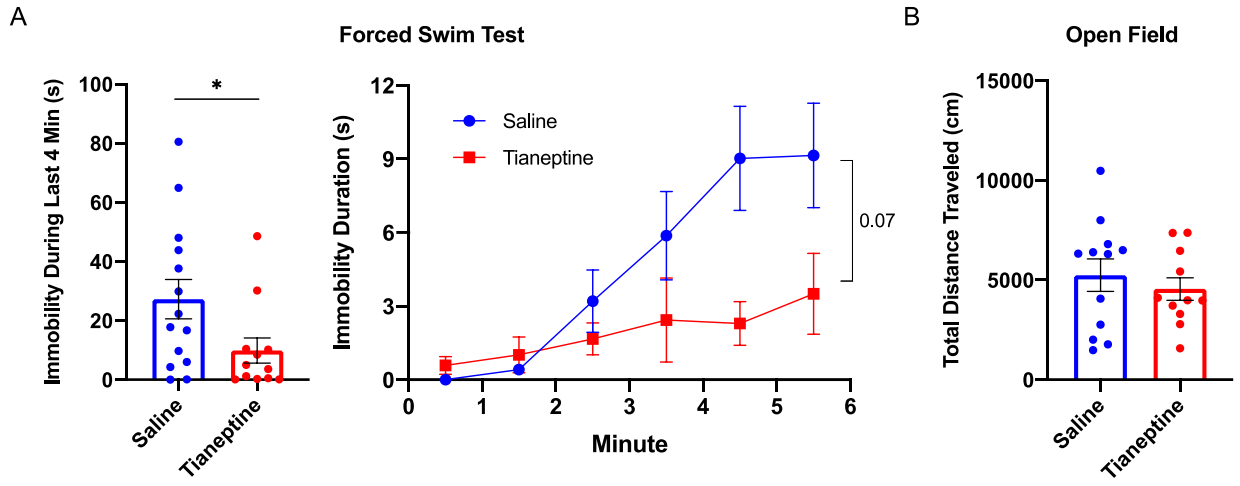

Figure 39: The ventral hippocampus may be sufficient to mediate tianeptine's acute antidepressant-like effects. Mice received infusions to the ventral hippocampus via bilateral cannulae targeted to Bregma-3.6, ML±2.8, DV-3.5. 15 minutes post infusion, they underwent FST, followed by OFT. A) Forced swim test results. (Left) Immobility during the last 4 minutes. Unpaired t-test: *p=0.0453. (Right) Immobility by minute for the entire 6-minute test. Repeated measures ANOVA: p=0.0749. B) Locomotion was assessed in the open field following FST to detect possible hyperactivity confounds. Unpaired t-test: p=0.5017.

decreased FST immobility compared to saline controls (Figure 39A), and that this difference could not readily be attributed to increased tianeptine-induced hyperlocomotion, as there was no statistically significant difference in open field locomotion (Figure 39B). This suggests that hippocampal MORs alone may be enough to mediate tianeptine's acute antidepressant-like effect, and that tianeptine's opioid-like hyperactivity effect is likely mediated by MORs in another brain region.

3.5.3 Potential Involvement of VTA MORs in the Antidepressant-like Effects of Tianeptine

Of course, as discussed at length in Chapter 1, the hippocampus is far from the only MOR- and GABA-expressing structure that may mediate tianeptine's antidepressant effects. Another such region would be the ventral tegmental area (VTA), which is known to be involved in reward processing. Given that anhedonia and reduced motivation are common symptoms of depression, structures like the VTA likely contribute to the pathophysiology and perhaps even the etiology of MDD[285]. Moreover, in animal models of depression, studies have shown that stress activates VTA dopamine neurons and stimulates dopaminergic transmission to its various limbic targets[286,287].

Particularly relevant for the case of tianeptine is the well-characterized modulation of dopaminergic reward pathways by VTA MORs. The classical model of opioid reward proposes that MOR hyperpolarizes local VTA GABAergic interneurons, thereby disinhibiting dopamine release from VTA neurons projecting into the NAc[297]. This specific role of GABAergic cells is consistent with the finding that in rodents, the majority of VTA neurons were reported to be either dopaminergic or GABAergic, and that MOR agonists inhibited GABA but not dopamine neurons[304]. Our own RNAscope images provide cursory support for the distribution of VTA MORs primarily on GABAergic cells, since RNA transcripts for MOR (*Oprm1*) and tyrosine hydroxylase (*Th*, a marker for dopaminergic cells) do not colocalize (Figure 40A), and MOR expression appears to be diminished in MOR-floxed VGAT Cre mice (Figure 40B), which, as established previously, are selectively deficient in GABAergic MORs (Figure 38A,C).

Finally, based on our behavioral data from SST Cre mice, we expect the brain region(s) mediating tianeptine's antidepressant-like effects to express MORs specifically on SST interneurons. Previous studies have shown that the VTA also has SST cells (most of which are

VGAT positive)[376]; thus, if MORs in the VTA are primarily expressed on GABAergic cells, it is likely that these SST cells would also have MOR, enabling the VTA to fit our criterion.

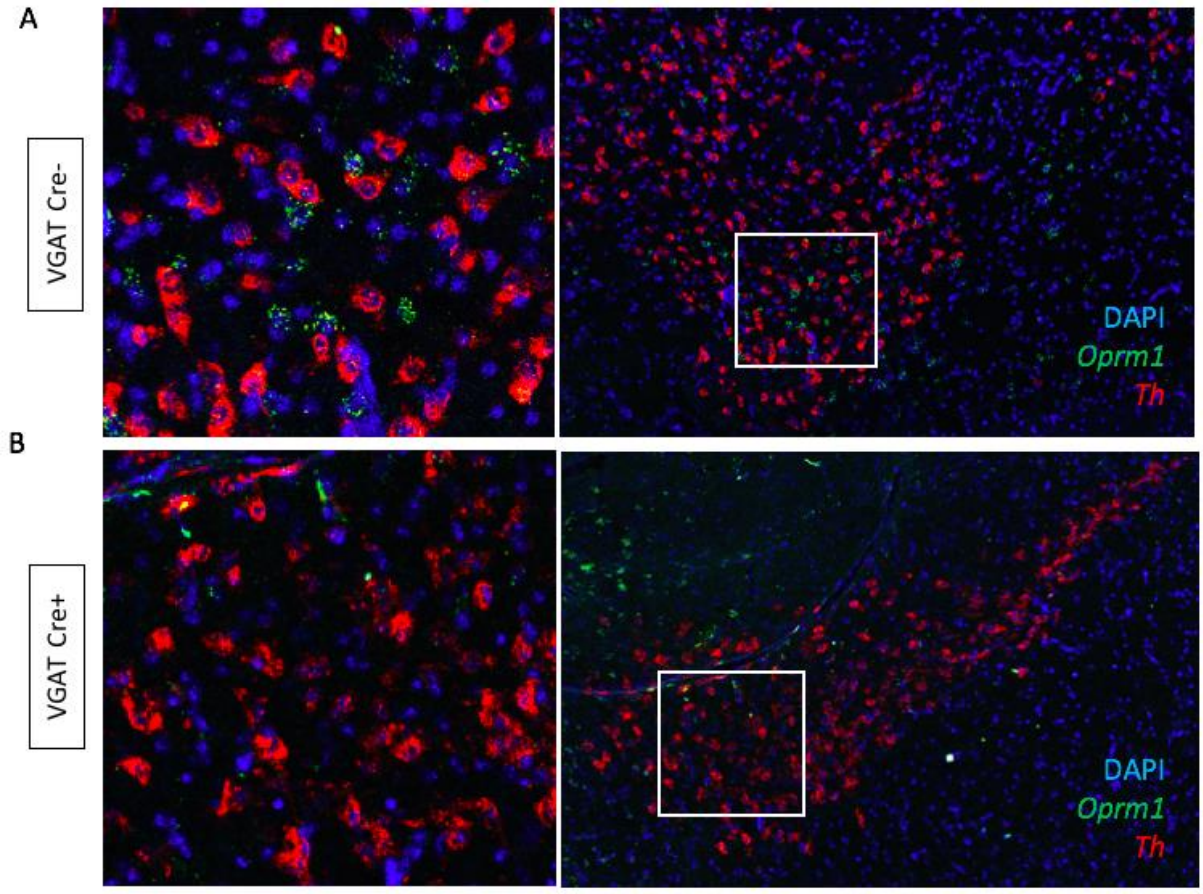

Figure 40: MOR (*Oprm1*) and tyrosine hydroxylase (*Th*) mRNA expression in the VTA. Representative confocal images double-labeling *Oprm1* (green) and *Th* (red) transcripts in the VTA of VGAT Cre- (A) and Cre+ (B) mice. A) MOR and TH appear to be expressed in somewhat discrete neuronal populations. B) At a glance, there appears to be less *Oprm1* expression in the VTA of VGAT Cre+ mice compared to Cre- controls. The images on the left are magnified views of the areas within the corresponding white square of the rightmost image. DAPI stain is shown in blue.

In fact, Alexander Harris has already done some intriguing preliminary work examining the role of VTA MORs in mediating the behavioral effects of tianeptine, which could inform some of the next steps for this thesis work. Harris showed previously that optogenetically inhibiting VTA GABA activity during restraint stress rescues reward-seeking, whereas activating

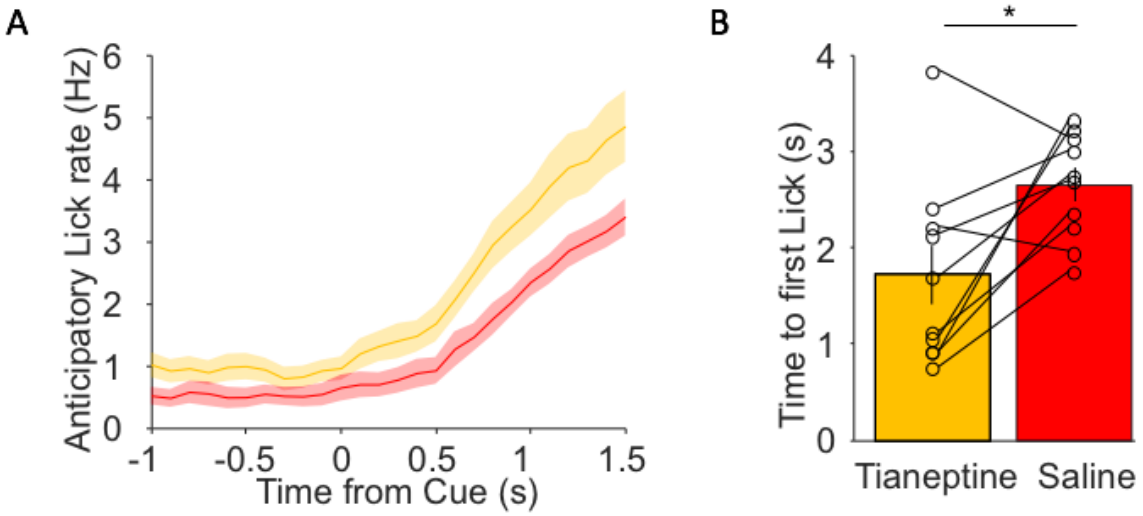

Figure 41: Tianeptine may restore reward-seeking behavior in acutely-stressed animals. Mice were injected with tianeptine (30 mg/kg, i.p.) or saline over 2 days. 1 hour post-injection, they were restrained for 30 minutes before engaging in a cued-reward task. A) Tianeptine (yellow) appears to increase anticipatory lick rate behavior in mice compared to saline (red). B) Tianeptine administration decreased the time to first lick during the reward availability period. (N=9), $p<0.05$ by paired signed rank test. Graphs reproduced with permission from Alexander Harris.

VTA GABA neurons in the absence of stress impairs reward seeking (personal communications). Thus, he sought to determine whether tianeptine, as an MOR agonist (coupled to Gi), could restore reward seeking behavior by inhibiting VTA GABA activity. Using a cued-reward task, he found that acute tianeptine administration (30 mg/kg i.p., over 2 days) partially restored reward seeking behavior following acute restraint stress, as evidenced by a trend towards increased anticipatory lick rate (Figure 41A) and significantly decreased time to first lick (Figure 41B) during the reward availability period. This suggests that tianeptine may be able to reverse stress-induced anhedonia-like behaviors in much the same manner as optogenetic inhibition of the VTA.

However, these results do not confirm that tianeptine's effects on reward are in fact mediated by VTA GABA neurons. Future work that repeats this behavioral task after local

infusion of tianeptine directly into the VTA must be done to definitively establish the validity of this proposed mechanism. This and other such experiments to probe the involvement of MORs in specific brain regions or cell types will be discussed in greater detail in Chapter 4.

Chapter 4: Discussion

4.1 MOR Modulation of Excitatory/Inhibitory Balance

Here, we demonstrate that MOR is required for the antidepressant-like behavioral effects of tianeptine following both acute and chronic administration, and use tissue-specific MOR knockout (via a floxed MOR mouse) to further show that MOR expression on a subset of GABAergic cells is necessary for these acute and chronic antidepressant-like responses. These results highlight the critical role of inhibitory neurotransmission, specifically in mediating tianeptine's behavioral effects, and possibly also in the development of and recovery from depression in general.

Research in recent decades has repeatedly highlighted how perturbations in the balance of excitatory and inhibitory signaling can promote depressive-like brain states [197,377]. This excitatory/inhibitory (E/I) balance arises from the interplay of glutamatergic pyramidal neurons and GABAergic interneurons interacting within a local circuit, and changes to these cellular components—e.g. through chronic stress—have been hypothesized to support the emergence of maladaptive clinical symptoms over time[378,379]. Both the glutamatergic and GABAergic systems have been extensively implicated in the pathophysiology of depression—as evidenced by studies of depressed patients[177,380] (including those with late-life depression[381,382]) and animals exposed to chronic stress[150]—and either could ostensibly provide the neuronal substrate for tianeptine's antidepressant effects.

Earlier research on tianeptine, prior to the discovery that it is an MOR and DOR agonist, speculated that its mechanism of action involved the glutamatergic system. A significant body of evidence supports a role for glutamate and its receptors—broadly divided into ionotropic receptors (including NMDARs, AMPARs and kainate receptors) and metabotropic glutamate

receptors (mGluRs)—in depression and antidepressant activity. In imaging studies, MDD is associated with an increase in neural activity/excitation in several brain regions believed to be critically involved in the pathophysiology of depression[383,384], and brain, plasma, and CSF glutamate levels all have been shown to be elevated in patients with MDD[385-387]. Additionally, post-mortem studies have reported alterations, both in glutamate receptor densities and in the expression or function of the NMDAR subunits, in individuals with depression or bipolar disorder, and in victims of suicide [388-392]. Moreover, glutamate release is highly sensitive to stress, and depressive-like states are frequently characterized by region-specific changes (generally increases) in the activity of glutamatergic pathways and enhanced activity at NMDA receptors[393,394]. Transgenic mouse models with modified glutamate system components exhibit changes in depressive-like behavior, and established animal models of depression demonstrate altered glutamatergic neurotransmission[395]. Some evidence from animal studies also suggests that antidepressants attenuate glutamate release in corticolimbic structures and modulate cortico-hippocampal populations of NMDA receptors in rodents[396,397]. Crucially, the rapid acting antidepressant ketamine is a non-competitive antagonist at the NMDAR, providing a clear precedent for a primarily glutamatergic mechanism of antidepressant action.

Multiple lines of evidence have specifically highlighted the interaction between tianeptine and the glutamatergic system. For instance, tianeptine, but not fluoxetine, has been found to attenuate stress-induced glutamate release in the basolateral amygdala. Tianeptine also prevents the reorganization of hippocampal glutamatergic synaptic vesicles in response to stress[152,398,399] and may thereby serve to normalize synaptic function. Because the glutamatergic system plays a key role in mediating neuroplasticity, it may also be the substrate

through which tianeptine reverses stress-induced structural alterations, including hippocampal neurogenesis, cell proliferation, and dendritic remodeling[150,219,398] (processes commonly disrupted in depressive states), and exerts its beneficial effects on cognition and memory[158,167,168,398,400].

However, in light of the finding that tianeptine interacts solely with MOR/DOR and is inactive at both ionotropic and metabotropic glutamate receptors[169], it is highly unlikely that tianeptine has a direct modulatory effect on glutamatergic signaling. Instead, tianeptine may restore normal glutamate neurotransmission and E/I balance by indirectly acting through opioid receptors and the GABAergic system. GABA itself has been extensively implicated in MDD: in general, diverse defects in GABAergic neurotransmission have been associated with depressive-like states in both patients and animal models (See Chapter 1 for detailed discussion). Thus it is conceivable that deficits in GABAergic signaling contribute to the etiology of depression by indirectly modulating glutamatergic signaling across several key brain regions known to be relevant in mood disorders, including the hippocampus, and that antidepressants may exert their therapeutic effects by reinstating proper GABAergic neurotransmission in these areas.

This is in line with our present findings that MORs on GABAergic cells are necessary for the acute and chronic antidepressant effects of tianeptine. At least within the hippocampus, MORs are primarily expressed on GABAergic interneurons, not on glutamatergic pyramidal cells themselves, as evidenced both by previous studies and by our own RNAscope images co-labeling VGAT and MOR (Figure 38). Moreover, acute treatment with opioid agonists (particularly mu selective ones) have been shown to increase net excitatory glutamate activity and facilitate long-term potentiation within the rodent hippocampus—an effect which can be blocked by GABA receptor agonists [401-403]. These observations support an indirect

hippocampal mechanism in which activation of MORs on GABAergic interneurons induces hyperpolarization and decreases GABA release, thereby increasing the excitatory activity of glutamatergic pyramidal neurons by disinhibition. In addition to acutely modulating GABAergic signaling, repeated stimulation of these pathways may normalize glutamatergic tone in the hippocampus , thereby explaining tianeptine's chronic antidepressant effects.

Although the experiments here focused exclusively on the antidepressant-like and opioid-like behavioral responses to tianeptine, it is interesting to note that tianeptine also produces marked pro-cognitive effects—as briefly mentioned earlier—since these further underscore the convergence of hippocampal GABA and opioid signaling in tianeptine's neurobiological mechanism. In rodents, tianeptine blocks stress- and ethanol-induced memory impairment and enhances performance in normal animals[400,404-406]. In depressed human patients, chronic tianeptine treatment improves memory and learning[144]. Animal studies have implicated both the hippocampus and MOR signaling in these effects. Lesioning excitatory inputs to the hippocampus impairs memory retention in rodents, and this deficiency can be reversed by tianeptine[407]. Moreover, pharmacologically ablating MOR in the hippocampal CA3 region impairs both memory acquisition and recall in rats[408]. The potential pro-cognitive effects of MOR agonists are also supported by the observation that GABA receptor antagonists, which mimic the disinhibitory effects of opioid agonists, improve both depressive and cognitive symptoms[409,410]. Thus, proper hippocampal memory formation may be contingent upon the release of endogenous opioids onto MORs on GABAergic interneurons. Given that cognitive impairment is itself commonly an axis of the depressive phenotype, future work on tianeptine's antidepressant mechanisms should specifically address this aspect.

Despite the overwhelming body of work addressing the dysregulation of GABAergic signaling in depression, a few crucial questions remain unresolved. GABAergic deficits are not unique to MDD, but are also implicated in numerous other neuropsychiatric disorders, particularly schizophrenia[411,412]. What distinguishes the GABAergic deficits among these different disorders? Moreover, how can we explain the limited use/efficacy of benzodiazepines (which produce their characteristic anxiolytic effect by engaging the $GABA_A$ receptor) in treating MDD? Although TCAs and benzodiazepines were initially both prescribed to treat depression, subsequent clinical studies and meta-analyses have established that the latter's antidepressant efficacy is limited to the triazolo-benzodiazepine alprazolam, which may match TCAs with respect to the anxiety, sleep, and anhedonia indices of depression, but fall short with regards to relieving depressed mood[413,414].

One explanation for this phenomenon is that benzodiazepines may interfere with the hippocampal neurogenesis, as co-treatment with the benzodiazepine diazepam was found to block the effects of chronic fluoxetine treatment on proliferation and survival of adult-born hippocampal neurons[415]. However, in this work we have presented evidence that unlike fluoxetine, chronic tianeptine does not promote hippocampal neurogenesis (Figure 17). Moreover, benzodiazepines are still often used in conjunction with standard antidepressants[416,417]. Taken together, these data suggest that neurogenesis is not a requirement for antidepressant efficacy, and that benzodiazepines (along with other GABAR-acting compounds) may still have niche therapeutic benefits in treating MDD. The drawbacks of tolerance and abuse potential for these drugs, which preclude them from prolonged use as antidepressants[418] may yet be ameliorated by the development of novel subtype-specific agonists of $GABA_A$Rs such as eszopiclone, which already shows promise as an antidepressant

for patients suffering from insomnia[419,420]. This is much the same trajectory that we hope to see with opioid-based antidepressant therapies, in which greater understanding of the nuances of receptor engagement and downstream signaling will help us to develop drugs that harness the therapeutic potential of these neurobiological systems while minimizing deleterious side effects.

4.2 MOR modulation of GABAergic Neuron Subtypes

4.2.1 SST Neurons

In addition to the general involvement of MORs on GABAergic interneurons, we specifically identified MORs on SST cells as the receptor subpopulation that is likely to be necessary for tianeptine's acute and chronic antidepressant-like effects in mice. This finding adds to a growing body of evidence implicating SST neurons in the pathophysiology and treatment of MDD (See Chapter 1).

SST is a modulatory and inhibitory neuropeptide that is largely co-localized with GABA, thus defining one of the three major cortical interneuron subtypes. It is involved in regulating multiple aspects of physiological and behavioral stress responses, such as the release of hormones from the hypothalamic-pituitary axis (HPA), and the cortical local circuit integration of sensory input. MDD patients have been found to exhibit decreased SST levels in the CSF, which can normalize with recovery[421,422], and human post-mortem studies have identified various region-specific SST deficits in depressed individuals, including downregulation of SST gene expression in the dorsolateral prefrontal cortex, subgenual anterior cingulate cortex and amygdala[177,202,423,424]. SST KO mice recapitulate several hallmarks of MDD, such as increased depressive- and anxiety-like behaviors, increased corticosterone, and reduced expression of brain-derived neurotrophic factor (BDNF)[425]. Acute inhibition of SST

interneurons in the PFC was also found to increase mouse behavioral emotionality, consistent with a central role for SST+ neurons in emotional regulation[425]. Interestingly, chronic blockade had the opposite effect, suggesting that network adaptations may recruit other brain regions/cell types in the long run[425]. SST cell function was also associated with fear learning and working memory in mice, whereas disruptions were associated with deficits in these dimensions[426,427]. Moreover, reduced SST cell function was associated with memory deficits in a mouse model of Alzheimer's disease[428]. Given tianeptine's known pro-cognitive effects, future research should incorporate behavioral tests that assess these dimensions in MOR-floxed SST Cre+ mice.

There is some evidence suggesting that SST plays a direct role in the development and treatment of mood disorders. In mice, intra-amygdalar and intra-septal microinfusions of somatostatin analogs produce anxiolytic effects in the elevated plus maze and shock-probe tests[429], and intra-cerebroventricular infusions of a selective Sst_2 or Sst_3 receptor agonists elicit antidepressant-like effects in the FST[207]. The effects of antidepressants on SST is not always straightforward, however. Chronic imipramine treatment increases SST expression in the mouse hypothalamus[430], but not in rats[431]. Similarly, chronic administration of maprotiline, mianserin, carbamazepine or zotepine does not affect SST levels in various rat brain regions[431,432], and the experiments examining the effect of chronic citalopram treatment on rat SST levels were inconclusive[432,433]. As such, for the purpose of this thesis, we solely considered SST as a marker for a discrete interneuron population, given the comparatively robust literature on GABAergic deficiencies in MDD, and the recent discovery that disinhibiting SST expressing interneurons (by specifically deleting the γ2 subunit of $GABA_A$Rs in these cells) produced an anxiolytic and antidepressant-like brain state[205].

Although our major finding that MORs on SST interneurons are required for the antidepressant-like effects of tianeptine seem largely congruous with the body of existing research, it may still be surprising to see that a relatively small population of cells mediates this behavioral response. SST-expressing GABAergic cells comprise about 30% of the cortical interneuron population, and the number of those neurons also expressing MOR is but a fraction of that (Figure 38B). However, it is by no means unprecedented for relatively few cells to produce significant behavioral effects, as can be seen in the seemingly disproportionate impact of a the small population of young adult-born hippocampal granule cells on overall dentate gyrus circuitry[434].

Another concern arises when we consider the direction of tianeptine's effects on MOR-modulated GABAergic signaling. Because MOR is an inhibitory $G_{i/o}$-coupled receptor, the body of literature claiming that GABA and SST deficits causally contribute to depression (see Introduction for details) seems at odds with our results that agonizing MOR on these interneurons (thus inhibiting these cells) has an antidepressant-like effect. There are, however, multiple explanations that could account for this apparent discrepancy.

For one, much of the work linking lower GABAergic transmission (often via SST interneurons) to depressive states involved whole brain manipulations, which could obscure the antidepressant contributions of modulating GABAergic inhibition in specific brain areas or cell types that are targeted by tianeptine. Studies have shown, for instance, that tianeptine can have diametrically opposed effects in the hippocampus and amygdala[159], and can produce bidirectional effects on Fos within the amygdala which depend on the current state of ongoing activation in the network[435]. This suggests that tianeptine administration may not simply lower GABA neurotransmission throughout the brain as might be predicted based on its agonism

of inhibitory MORs on interneurons, but rather have more nuanced effects that depend on cellular context. If, for instance, tianeptine preferentially targets regions like the hippocampus—where it has indeed been found to increase excitability and plasticity[157,167]—its net excitatory effect there would be consistent with an antidepressant-like effect, as reduced activity of the hippocampus and other forebrain structures has been observed in depressed patients[436-438] and reported to be reversed by antidepressant treatment [439,440].

Moreover, tianeptine is not the only antidepressant that is thought to involve pyramidal cell disinhibition via suppression of SST-cell mediated GABAergic signaling. Scopolamine, a competitive inhibitor of the muscarinic acetylcholine receptor (mAChR), produces rapid and lasting antidepressant effects, which emerge within 3 days and persist for about a week (a duration that can be prolonged by repeated administration), even in otherwise treatment-resistant patients[441]. In mice, the antidepressant-like effects of scopolamine were found to depend in part on mAChR antagonism specifically in SST cells of the medial prefrontal cortex[187].

Ketamine, a noncompetitive NMDAR antagonist and the posterchild of rapid antidepressants, has also been suggested to exert its antidepressant effects via GABAergic disinhibition of glutamate signaling[442]. Interestingly, spiking signature analysis and studies using the selective NR1/NR2B receptor antagonist ifenprodil have found that that NMDA receptors appear to be enriched in putative SST interneurons, suggesting that this population of interneurons may be particularly relevant for the effects of ketamine and other NMDA-selective compounds[443].

4.2.2 D1 and D2 MSNs

We considered the possibility that MORs on D1 medium spiny neurons (MSNs) were the receptor subpopulation mediating tianeptine's antidepressant-like effects largely based on work by Cui *et al.* Using a conditional bacterial artificial chromosome (BAC) rescue strategy, they demonstrated that targeted expression of MOR on D1 MSNs is sufficient to restore morphine-induced CPP reward and striatal dopamine release—both of which are absent in MOR KO mice[310]. Since anhedonia is a core symptom of MDD that may reflect an underlying dysregulation in reward processing, we expected that MORs on D1 would be necessary for tianeptine's antidepressant-like and rewarding effects.

We found, however, that tianeptine was still able to reduce forced swim immobility and produce CPP in mice lacking MORs on D1 cells. Tianeptine's antidepressant efficacy in mice lacking a receptor subpopulation previously shown to be crucial for mediating opiate reward may seem surprising, but it is in fact consistent with our MOR-floxed VGAT Cre data, which decoupled antidepressant-like effects from CPP reward. Much more perplexing is the result that tianeptine-induced CPP development remains intact in mice lacking D1 MORs, as this appears to directly contradict Cui *et al*.'s results.

There are several explanations that may help account for this incongruity. For one, the experimental approaches in the two studies were complementary but not necessarily equivalent: Cui *et al*. tested the *sufficiency* of MORs on D1 MSNs for mediating opiate reward by re-expressing MORs in that specific subpopulation, whereas we looked at the *necessity* of D1 MORs by knocking out MORs in that cell type. It is therefore possible that D1 MORs might be fully capable of reinstating opiate reward, but that in their absence, other brain regions/cell types can compensate so that no behavioral changes are observed. Future experiments that address the

sufficiency question by locally infusing tianeptine into the striatum prior to behavioral testing could be done to determine if this is likely to be the case.

Additionally, there are some differences in the exact population of D1 MORs addressed by the two studies, which may also contribute to the discrepancy in results. Cui *et al*. used the GENSAT mouse *Pdyn* (encoding prodynorphin) BAC (RP23-358G23), which drives transgene expression in a relatively restricted pattern in the striatum[444], to re-express MOR in their MOR KO mice, while our MOR-floxed mice were bred with D1-Cre mice (MGI:3700228), which show Cre- activity in all the major brain regions known to express D1, including the NAc, dorsal striatum, amygdala, hippocampus, and PFC[445]. Because our genetic manipulation was broader (and constitutive), there may be a higher chance that brain-wide adaptations occurred to compensate for the loss of MOR. Alternatively, D1 MORs in these various brain regions may have opposing effects, such that knocking them all out obscures the contribution of each specific receptor subpopulation.

Along those same lines, it may be useful to consider the role that may be played by MORs on the other major MSN subtype: D2 neurons. There are clear anatomical and functional distinctions between these two neuronal groups. NAc D1 MSNs send direct projections back to the VTA, as well as to the substantia nigra and ventral pallidum (VP), while D2 MSNs signal exclusively through the VP, thereby forming the direct and indirect pathways, respectively[308]. Although MORs are preferentially expressed on D1 compared to D2 MSNs, they are still present in the latter population[446,447], and so their potential contributions should not be ignored.

Moreover, D1 and D2 MSNs have been shown to play an important, if somewhat different, roles in reward- and depression-related behaviors. Optogenetic stimulation of D1 MSNs was found to acutely increase the rewarding effects of cocaine[448], and to promote

resilience following social defeat stress[449]. Daily stimulation of D2-MSNs during the chronic social defeat stress paradigm, by contrast, promoted social avoidance over a longer time span[449]. Remarkably, several classes of antidepressants have been found to increase D2 mRNA and/or receptor occupancy in the NAc, suggesting that D2 upregulation may also influence the normalization of hedonic tone in patients with MDD. It should be noted, however, that there is also evidence of MSN subpopulations co-expressing both D1 and D2 receptors[450], whose role in in anhedonia and depression remains unclear. Future research looking specifically at the role of MORs on D2 cells (using D2-Cre mice, MGI:3836635) or MORs on all MSNs (with RGS9-Cre mice, MGI:3692442) remains to be done.

4.2.3 PV and 5-HT3a/VIP Interneurons

Finally, we would be remiss not to include a brief mention of the other two major GABAergic subpopulations: PV- and 5-HT3a/VIP-expressing interneurons. The evidence surrounding the role of these two interneuron classes in MDD is less consistent than for SST[380,423], but there are certainly some compelling data implicating PV cells in stress and depression. For instance, reduced expression of PV has been observed in the ACC of MDD patients[202], and PV cells were found to be susceptible to the effect of prolonged psychosocial stress in tree shrews[451]. While we found no evidence that MORs on PV cells were required for the acute antidepressant-like (nor any other behavioral) response to tianeptine, we cannot wholly discount the potential contributions of MOR-modulated PV+ GABAergic signaling, as 1) the behavioral tests we conducted do not reflect the full spectrum of possible depressive symptoms (for instance, the cognitive or motivational dimensions of MDD), 2) we never assessed the possibility that chronic but not acute tianeptine requires MORs on PV interneurons, and 3) it is

possible that tianeptine acts on multiple interneuron subpopulations simultaneously, such that one subset of MORs could compensate for the depletion of another, or have directly opposite effects. VIP interneurons for instance, inhibit both SST and PV cells, and a minority of SST cells are known to provide inhibitory input to PV cells.

Of course, future experiments should certainly be conducted to determine whether MORs on VIP/5-HT3a-expressing cells are necessary for the behavioral effects of tianeptine, which we did not at all address in this thesis. This investigation of this population was omitted for the time being, due to a lack of convenient Cre mouse line. Unlike for PV and SST, generating an MOR floxed +/+ VIP Cre mouse proved impossible because the VIP and MOR genes were located at nearly adjacent loci in our MOR-floxed and VIP Cre (Jackson Stock No: 010908) mouse lines.

4.3 Brain Regions

Although we have elucidated which cell types may be necessary for tianeptine's antidepressant-like effects, it is less clear which brain regions are involved. We demonstrate that the medial habenula, one of the strongest expression sites for MORs[281], is likely not a site of action for tianeptine, as tianeptine produces a robust antidepressant-like response in mice lacking a large fraction of habenular MORs, and both MOR-floxed VGAT-Cre+ and MOR-floxed SST-Cre+ mice (which do not respond to tianeptine) show no decrease in MOR RNA expression in the habenula.

Instead, we speculate that MORs in hippocampal neurons may be responsible for tianeptine's acute and chronic antidepressant effects. Not only does the hippocampus have high expression of MORs on GABAergic and SST+ cells, it also undergoes dramatic changes during

depression, including dendritic atrophy, decreased volume, reduced levels of cerebral metabolites, and decreased adult neurogenesis[130,214-216]. Most compellingly, many of the morphological changes to the hippocampus observed in depressed/chronically stressed subjects can be reversed specifically by tianeptine. In tree shrews, tianeptine prevents changes in hippocampal volume, cell proliferation [150], and apoptosis in the dentate gyrus[219] following psychosocial stress. In rodents, tianeptine reverses and prevents the stress- and corticosterone-induced atrophy of CA3 pyramidal neurons in the hippocampus[153,220], while fluoxetine does not [152].

As mentioned previously, the hippocampus is also a structure where the net excitatory effect of tianeptine is consistent with what is known about the pathophysiology of depression. Our data support the notion that the hippocampus may be an important site of action for tianeptine, as we find that expression of MOR on GABA and SST cells is significantly reduced in the ventral hippocampi of MOR-floxed VGAT-Cre+ and MOR-floxed SST-Cre+ mice respectively, compared to their WT littermates. Tianeptine's lack of efficacy in these Cre+ mice could potentially be explained by this deficit, although experimental manipulations must be done to more directly test this hypothesis. Specifically, future studies could assess whether locally knocking out hippocampal MORs via adeno-associated virus (AAV)-Cre injections into the hippocampi of MOR-floxed mice is enough to prevent the antidepressant-like and/or opioid-related effects of tianeptine treatment.

However, this proposed experiment, too, is limited in that it only addresses the brain area, and not also the cell type involved in MOR mediation of tianeptine's behavioral effects. In order to unambiguously assert that, say, MORs on GABAergic cells in the hippocampus are necessary for tianeptine's therapeutic efficacy, a more complex experimental design is necessary. One

possible intersectional approach could utilize another site-directed recombination technology (Flp-FRT), which involves the recombination of sequences between flippase recognition target (FRT) sites by flippase (Flp) recombinase (Flp). Crossing MOR-floxed mice to mice expressing Flp under a VGAT promoter (e.g. Jackson Stock No: 029591) would produce MOR-floxed mice that only express Flp in GABAergic interneurons. Viral injection of a flippase-dependent flirted Cre (in which Cre is flanked by FRT sites) into the hippocampus of these mice would selectively knock out MOR in hippocampal GABA interneurons. The standard battery of behavioral tests used throughout this thesis could then be implemented to determine whether interneurons in the hippocampus are necessary for tianeptine's antidepressant- and opioid-like effects.

Alternatively, we could also take advantage of a newer method which uses virally delivered Cre-dependent CRISPER/Cas9 to mediate conditional gene modification in the brain. The RNA-guided Cas9 endonuclease, from the prokaryotic clustered regularly interspersed short palindromic repeats (CRISPR) adaptive immune system, has enabled scientists to achieve site-specific genome editing of eukaryotic cells[452]. Viral vector-based delivery of CRISPR/Cas9 was initially hampered by the large size of *Staphylococcus pyogenes* Cas9 (SpCas9), which required separate vectors for the delivery of the SpCas9 and corresponding single guide RNA (sgRNA)[453,454], but these problems were largely mitigated by the characterization and use of the smaller *Staphylococcus aureus* Cas9 (SaCas9)[455,456]. Indeed, it has recently been demonstrated that an AAV vector containing a Cre-dependent SaCas9 and an sgRNA can mediate cell type-specific mutagenesis are as efficiently as conventional conditional gene knockout[457]. As such, we could selectively inactivate MOR in hippocampal GABAergic cells by cloning an sgRNA selected for MOR into pAAV-FLEX-SaCas9-sgRNA (containing an

inverted SaCas9 flanked by two sets of staggered loxP sites for Cre-mediated inversion/excision) and injecting the vector into the hippocampus of VGAT Cre mice.

Of course, all of these procedures can be done to examine the role (with or without cell-type specificity) of not just the hippocampus, but any other candidate region for mediating tianeptine's antidepressant- and opioid-like effects (for detailed discussion of various brain structures, see Chapter 1). Nevertheless, it would perhaps be advisable to start with AAV-Cre viral injections into the VTA of MOR-floxed mice. Because MORs are almost exclusively expressed on GABAergic cells in the VTA, this procedure would provide some insight into cell-type specificity without having to implement any of the more complex experimental methods, which have yet to be piloted in the lab.

4.4 Opioid Antidepressants: Theoretical Implications and Clinical Outlook

4.4.1 The Nature of Opioid Antidepressants

Broadly, this work has intriguing implications about the nature of opioid antidepressants. Two overarching hypotheses that have been used to justify the use of opioids as a treatment for depression are euphoria (i.e. that the rewarding effects of opioids counteract anhedonia)[22] and mental pain (i.e. that the putative overlap between the circuits underlying physical and mental pain means that analgesics may also help alleviate aversive emotional states)[458]. However, our results do not directly support either of those notions, as both conditioned place preference and hot plate analgesia have been dissociated from acute antidepressant-like effects for tianeptine. This does not mean that the reward and pain systems are irrelevant to the pathophysiology of depression, as depression is a heterogenous disease that manifests differently and may involve distinct neurobiological dysregulations in different individuals. However, it does suggest that

these two circuits may not be the ones responding to tianeptine in a manner captured by our current depression assays.

It is as of yet unclear what networks are engaged by tianeptine, but we can speculate about the possibilities. For instance, instead of producing euphoria or reducing pain, tianeptine might work by restoring proper executive function and mood by engaging structures such as the prefrontal cortex (PFC), anterior cingulate, hippocampus, and amygdala, all of which are interconnected and have been shown to exhibit morphological and functional abnormalities in depressed patients[459].

4.4.2 Clinical Potential of Tianeptine

One of the major findings of this thesis work is that tianeptine has a distinct mechanism of action than SSRIs like fluoxetine, in three crucial regards: 1) tianeptine does not directly engage the serotonin system by interacting with any known serotonin receptors or transporters, nor is its acute antidepressant-like efficacy hampered by partial serotonin depletion, 2) tianeptine requires MOR but not DOR expression for its antidepressant-like effects, whereas fluoxetine is the opposite, and 3) unlike fluoxetine, tianeptine does not promote the proliferation or survival or adult-born neurons in the hippocampus, nor does it require neurogenesis for its chronic antidepressant effects. To our knowledge, this is the first time a direct distinction has been drawn between tianeptine and SSRIs. Notably, we also show that tianeptine produces an antidepressant-like phenotype in mice after just one week of treatment, which is in line with at least one clinical study which reports initial therapeutic benefits after one week of treatment with tianeptine, rather than several weeks as required for SSRIs[140].

Due to this distinct mechanism, tianeptine might be effective in specific subsets of patients for whom current treatments are suboptimal, such as the patients who are elderly[145], have depression refractive to SSRI monotherapy[146], or have depression comorbid with other disorders such as Parkinson's[147], PTSD[148] or alcohol addiction[149]. In particular, depressed patients with high rejection sensitivity (sometimes called atypical depression) may be uniquely situated to benefit from mu opioid-based antidepressant treatments, as rejection sensitivity has been recently associated with opioid deficits[460]. Depressed patients display reduced MOR activation in brain regions regulating stress, mood, and motivation during social rejection compared to healthy controls[111], and a functional variation of the MOR gene has been linked to dispositional and neural sensitivity to social rejection in humans[108]. In fact, a clinical trial testing whether chronic tianeptine treatment improves depressive and rejection-sensitive symptoms in such individuals is currently being conducted by the Hope for Depression Research Foundation (HDRF).

4.4.3 Tianeptine and Suicidality

Although research in this area is quite limited, tianeptine may also compare favorably with other antidepressants with regards to preventing the development and worsening of suicidal ideation. Depression and suicidal behavior are intimately linked: 40–80% of suicide attempts are directly linked to a depressive episode[461] and suicide rates range from 5 to 20% among depressed patients [462]. However, antidepressants are surprisingly ineffective in depressed patients at high risk of suicidality[463,464], and poor response to antidepressant treatment can predict worsening of suicidal risk[462]. In fact, some patients even experience treatment emergence or worsening of suicidal ideation (TESI and TWOSI) after antidepressant initiation[465].

It is unclear whether the risk of TESI or TWOSI differs depending on specific antidepressant mechanisms, but there is notably an emerging body of research implicating the opioid system (especially MORs) in suicidal behavior. Patients using high doses of opioids seem to be more inclined toward suicidal ideation and to attempt suicide[466-468], and pronounced increases in the concentration, but not the affinity, of MORs in the prefrontal cortex, temporal cortex, and the basal ganglia has been observed in the post-mortem brains of suicide victims, possibly as a consequence of a compensatory mechanism[469,470].

Moreover, recent studies have demonstrated that very low doses of buprenorphine (a partial mu agonist and kappa antagonist) decreased suicidal ideation in suicidal patients[128] and in depressed patients with comorbid opiate addiction[471]. It has been hypothesized that buprenorphine's "anti-suicidal" effect is mediated by its mu agonism whereas its antidepressant effect hinges on its kappa antagonism[472]. A recent study found that polymorphism A118G on MOR is associated with emergence of suicidal ideation at antidepressant onset in a cohort of depressed outpatients being treated with tianeptine[473], further implicating MORs in the pathophysiology of suicidal behaviors. Most strikingly, tianeptine has been found to be significantly associated with a lower risk of suicidal ideation worsening compared with other antidepressants in the first 6 weeks of treatment, suggesting that opioid agonists may help reduce the risk of worsening of suicidal ideation at antidepressant onset[465].

4.4.4 Potential Risk of Tianeptine Abuse

Tianeptine's opioid-based mechanism may raise concerns about its potential abuse liability, but we have shown previously that tianeptine has a short half-life and displays a reduced withdrawal/tolerance profile compared to other mu-opioid agonists (Figure 20). Case

reports of tianeptine dependence and withdrawal predominantly feature individuals with a prior history of substance use disorder who had been taking far more than the recommended therapeutic dose[474]. As such, while tianeptine is certainly not without its risks, given proper medical supervision, it may still be a viable treatment option for certain populations of depressed patients.

Moreover, in addition to helping us identify the MOR subpopulations mediating tianeptine's behavioral effects, investigation of our various MOR-deficient mouse lines (MOR-floxed VGAT, D1, SST, and PV Cre) has also enabled us to demonstrate a double dissociation of the antidepressant-like phenotype from other opioid-like phenotypes resulting from acute tianeptine administration. While mice lacking MOR expression on GABAergic neurons failed to show the antidepressant-like effect, these mice still showed acute hyperlocomotion, analgesia, and conditioned place preference. Conversely, knockdown of MOR expression on other neuronal subsets resulted in an absence of typical opioid-like phenotypes, with an intact antidepressant-like phenotype. This suggests that tianeptine engages multiple circuits containing diverse cell types, and that the circuitry involved in producing antidepressant like-effects is likely distinct from those producing specific opioid-like effects. Thus, it may possible, through careful and targeted drug design, to develop novel therapeutics that engage "antidepressant" promoting circuits without producing opioid-related adverse side effects.

Conclusion

In conclusion, we have demonstrated that tianeptine is mechanistically distinct from SSRIs, both in its engagement of the mu opioid rather than the delta opioid or monoaminergic system, and in the hippocampal neurogenesis-independent nature of its chronic antidepressant-like effects. In doing so, our work also highlights the promising clinical applications of tianeptine for subpopulations of MDD patients who respond poorly to SSRIs. Furthermore, we have identified MORs on GABAergic—and more specifically, SST-expressing—neurons as the functionally relevant targets for tianeptine's acute and chronic effects, thereby illuminating a new avenue for understanding what circuit dysregulations may occur in depression and identifying an entry point for the development of new classes of antidepressant drugs.

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